Structure and virulence of *Legionella pneumophila* populations from freshwater systems in Germany and Middle East

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Summary

Legionella pneumophila is a bacterial pathogen present in natural and man-made freshwater environments that can cause a severe, and often fatal, pneumonia called Legionnaires' disease. Molecular epidemiology based on the application of typing methods is essential to determine the genetic relatedness between bacterial strains and understand the transmission and evolution of this pathogen. This thesis aimed to contribute to a more profound understanding of the genetic diversity of *L. pneumophila* strains and its clonal populations in different habitats and biogeographic regions. Furthermore, it aimed to get insights into the growth physiology and virulence of the most relevant clonal representatives. For this purpose, the high resolution genotyping method Multi Locus Variable number of tandem repeats Analysis (MLVA) was established and validated by comparison with the standard Sequence Base Typing (SBT). A total of 133 clinical and 478 environmental isolates of *L. pneumophila* from Germany, Israel and the West Bank were subjected to serological analysis and MLVA genotyping. Since temperature is one of the major factors that affect the growth of *L. pneumophila* in water systems, the growth in liquid medium of a subset of 63 isolates was examined at a range of temperatures from 15°C to 45°C in order to further understand the role that plays this environmental parameter in the physiology of clinical and environmental strains. Finally, the virulence of 85 strains was assessed by various in-vitro assays using THP-1 human macrophages like-cells.

MLVA genotyping presented higher discriminatory power than SBT yet, both methods were highly congruent. Genotyping analysis revealed the clonal nature of the populations of *L. pneumophila*. At genotype level high genetic diversity of the strains was observed. Only three MLVA genotypes, were common between Europe and the Middle East and only two were shared by Israel and the West Bank despite of the proximity of the two areas. Among the common genotypes, genotype Gt4(17) was generally the most frequently isolated and corresponded to the globally widespread Sequence Type 1 (ST1). However, the great majority of isolates from the different areas were genetically related as they appeared enclosed in VNTR Analysis-based clonal complexes (VACC). Analysis of growth at different temperatures revealed that the multiplication of clinical strains was enhanced at high temperatures (30ºC, 43ºC) in comparison to environmental strains, which appeared to be more adapted to lower temperatures (15ºC, 22ºC). In addition, temperature influenced the growth of specific genotypes differently. In particular, genotype Gt4(17) exhibited higher ability to grow at colder temperatures. The assessment of the pathogenicity in human macrophages indicated the high virulence potential of certain genotypes and clonal complexes. Overall, these findings may assist the understanding of the epidemiology of *L. pneumophila* strains and provide insights into the ecology of this opportunistic pathogen.

Zusammenfassung

Legionella pneumophila ist ein pathogene Bakterienart, die in natürlichen und technischen Süßwassersystemen vorkommt und schwere, oft tödliche, Lungenentzündungen verursacht, die Legionärskrankheit genannt werden. Für die molekulare Epidemiologie von *L. pneumophila*, die auf der Anwendung von Typisierungsmethoden basiert, ist es wichtig die genetische Verwandtschaft zwischen einzelnen Stämmen zu bestimmen, um seine Übertragungswege und Evolution zu verstehen. Die vorliegende Doktorarbeit zielt darauf hin ein tieferes Verständnis der genetischen Vielfalt von *L. pneumophila* Stämmen zu erlangen und ihre klonalen Populationen in verschiedenen Habitaten und geographischen Regionen zu bestimmen. Darüber hinaus sollte die Wachstumsphysiologie und Virulenz der relevantesten klonalen Repräsentanten bestimmt werden. Zu diesem Zweck wurde die hochauflösende Genotypisierungsmethode MLVA (Multi Locus Variable number of tandem repeats Analysis) etabliert und validiert und mit der gegenwärtigen Standardmethode SBT (Sequence Base Typing) verglichen. Insgesamt wurden 133 klinische und 478 Umweltisolate von *L. pneumophila* aus Deutschland, Israel und der West Bank serotypisiert und mit MLVA genotypisiert. Die Temperatur stellt für *L. pneumophila* einen der wesentlichsten Faktoren dar, die sein Wachstum in aquatischen Systemen beeinflussen. Daher wurde das Wachstum von 63 Isolaten in Flüsskultur im Temperaturebereich von 15°C bis 45°C untersucht, um die Rolle der Temperatur für die Physiologie von klinischen und Umweltisolaten aufzuklären. Abschließend wurde die Virulenz einer Reihe von Stämmen mit Hilfe verschiedener in-vitro Assays basierend auf humanen Zellkulturen (THP-1 human macrophages like-cells) bestimmt.

Die auf MLVA-basierende Genotypiserung zeigte eine höhere Unterscheidungsfähigkeit als die SBT-basierte, allerdings waren beide Methoden in hohem Maße kongruent. Auf der Ebene einzelner Genotypen wurde eine hohe genetische Diversität festgestellt. Nur drei MLVA Genotypen wurden sowohl in Europa als auch im Nahen Osten gefunden; nur zwei Genotypen fanden sich gemeinsam in Israel und der West Bank, trotz der großen räumlichen Nähe. Der Genotyp Gt4(17) wurde überall am häufigsten isoliert und entspricht auch dem global verbreiteten Sequenztypt 1 (ST1). Darüber hinaus bestand der Großteil der Isolate aus genetisch verwandten klonalen Komplexen. Die Analysen des Wachstums bei verschiedenen Temperaturen zeigten, dass das Wachstum der klinischen Stämme bei hohen Temperaturen (30-43°C) im Vergleich zu Umweltstämmen erhöht war, während Letztere besser bei niedrigen Temperaturen wuschen (15-22°C). Die Temperatur beeinflusste das Wachstum verschiedener Stämme eines spezifischen Genotyps unterschiedlich. Besonders der Genotyp Gt4(17) wuchs besser bei kalten Temperaturen. Die Bestimmung der Virulenz zeigte ein hohes Virulenz-Potential bei bestimmten Genotypen und klonen Komplexen. Insgesamt führen diese Ergebnisse zu einem umfassenderen Verständnis der Epidemiologie und Ökologie von *L. pneumophila* Stämmen.

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Chapter I

General Introduction

1.1 *Legionella pneumophila*: environmental bacterium and opportunistic pathogen

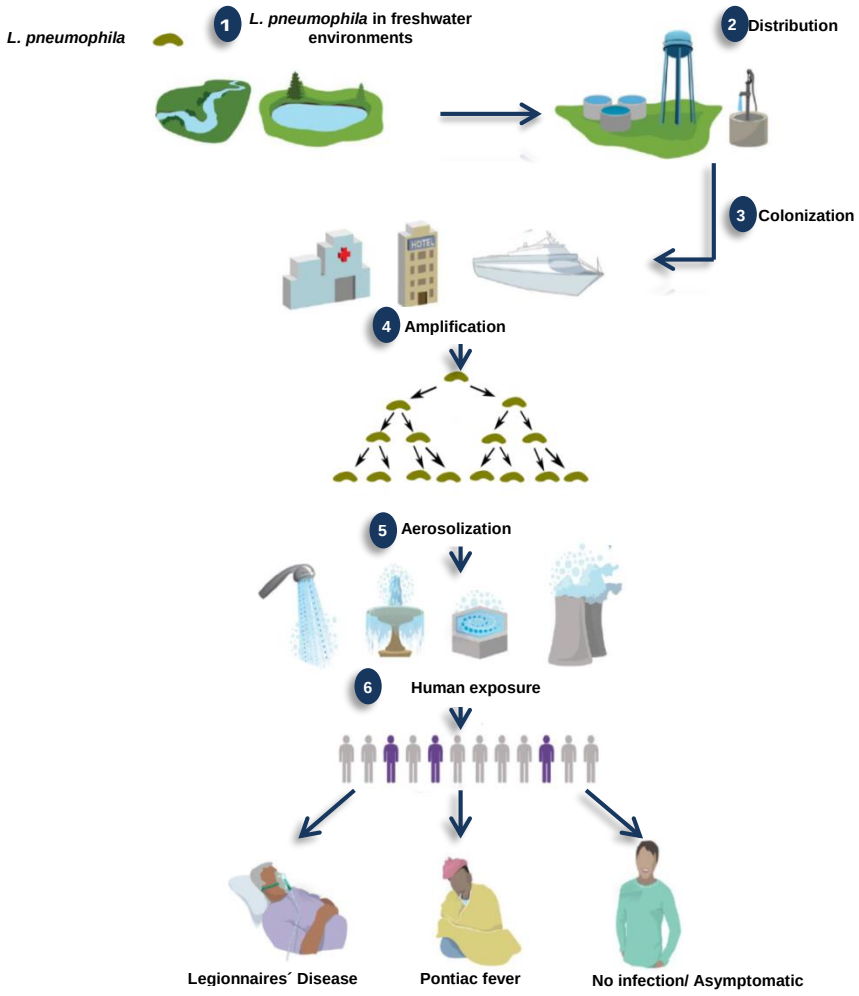
The provision of safe drinking water has been one of the most successful developments in public health of all times. Yet, inappropriate management of the drinking water supply systems (DWSS) and ignorance of the potential risks can lead to waterborne single infections and disease outbreaks. Some microorganisms, defined as opportunistic premise plumbing pathogens, are responsible for a significant number of waterborne infections and represent an emerging disease problem with major economic costs (1). *Legionella* species are one of the leading causes of drinking water associated individual infections and outbreaks, accounting for 58% of the cases in 2010 in the United States (2).

The genus *Legionella* was first described during the investigation of a major pneumonia outbreak among members of the American Legion attending a convention in Philadelphia in 1976. The causative agent was identified as a Gram-negative bacterium that was consequently named *Legionella pneumophila.* The name given to the infection was Legionnaires´ disease (LD), which denotes the pneumonic form of legionellosis. There is a non-pneumonic form of legionellosis, called Pontiac fever, which is a milder and non-fatal infection that usually goes undiagnosed due to lack of consensus on diagnostic criteria. After isolating the bacterium for first time many serogroups of *L. pneumophila* and other *Legionella* species have since then been discovered. Currently, there are 59 different *Legionella* species and 70 serogroups described and all can be considered potential human pathogens (3).

LD is an atypical pneumonia, whose incubation period is roughly 2–14 days and the symptoms range from mild disease to severe pneumonia that require hospital admission. Transmission is usually by inhalation of contaminated aerosols with *Legionella* spp generated by showers, faucets, air-conditioning systems or cooling towers (**Figure 1**). The amount of bacteria needed to result in disease, the infectious dose, is still not clear (4). It is thought that no person-to-person transmission exists. However, recently the first probable case of person-to-person transmission has been reported (5). Of all *Legionella* species, *L. pneumophila* (particularly, *L. pneumophila* serogroup 1) is the most virulent and most the common cause of disease, accounting with 87% of culture-confirmed cases of LD in Europe and 70-80% in United States (6, 2). The global incidence of LD worldwide is unknown and it is likely to be under-recognised, primarily because countries differ in diagnosis methods, common definitions

and reporting rates. In 2013, 5851 cases of Legionnaires' disease were reported to the European Legionnaires' Disease Surveillance Network, with the highest number of reported cases located in France, Italy and Spain. LD shows a seasonal pattern, having higher prevalence in the late summer to autumn. Some studies have associated this increase to warm and humid weather conditions (7, 8).

Figure 1. Route of distribution of *L. penumophila* from its natural freshwater habitats to the development of disease. *L. pneumophila* that inhabits freshwater environments (**1**) is spread through units of water treatment and purification plants (**2**) and ends up colonizing downstream water supply networks (**3**). Under appropriate environmental conditions the bacteria multiply (**4**) and disseminate by means of small aerosols droplets. (**5**). Aerosols reach the human population, which include individuals with different susceptibility (**6**). The outcome of the infection depends on the susceptibility of the individuals. [Modified from Mercante & Winchell 2015 (3)].



One of the major concerns about LD disease is the occurrence of outbreaks that affect a high number of people, including fatal cases. In the late summer of 2013, a large outbreak of LD occurred in the German city of Warstein. An industrial cooling tower was the source of the outbreak, which ended with 159 suspected and 78 laboratory-confirmed cases of LD, including

one death (9). In November 2014, again an industrial wet cooling system was the source of a LD outbreak in Northern Portugal, in which a total of 334 cases were confirmed, including ten deaths (10). The most recent LD outbreak in Europe took place in December 2015 in Spain. This outbreak accounted for 237 cases were laboratory-confirmed and four deaths. Unlike the previous outbreaks, in this case the source of infection seemed to be an ornamental fountain. The case-fatality ratio of LD has been reported in average as 10% (6). Yet, it might be higher in elderly people and people who have pre-existing medical conditions or smoke. Antibiotic resistance has never been reported for *L. pneumophila* and the treatment in case of infection are the commonly prescribed antibiotics recommended for other community and hospital-acquired infections of Gram-negative bacteria (e.g. macrolides or fluoroquinolones) (11).

1.2 Ecology and life cycle of *L. pneumophila*

L. pneumophila are aerobic *Gammaproteobacteria* that inhabit natural and anthropogenic freshwater environments. They have been found in rivers, lakes, hot springs and subsurface waters. However, infections are always traced back to man-made freshwater systems, such DWSS, cooling towers, whirlpool spas or even decorative fountains (12). Due to the low concentrations of nutrients in their oligotrophic natural habitats, *L. pneumophila* has adapted to live in biofilms, where they can obtain amino acids and carbon sources that they need for survival and replication. In biofilms, they are part of complex microbial communities (13) where they are subjected to predation by protozoa (14). However, living in the constricted environment of biofilms has evolved in the development of strategies to parasitize and multiply inside numerous protozoa species. They are able to parasitize at least 20 different species of amoeba, two species of ciliated protozoa, and one slime mold (15, 16), but are associated most frequently with amoeba belonging to the genera *Acanthamoeba, Hartmanella,* and *Naegleria* (17). The association with amoeba, in the cyst or the vegetative form, or just the presence in the biofilm, entail numerous benefits for *L. pneumophila*, such as protection from bactericidal or harsh environmental conditions (high temperatures, acidity, high osmolarity, chlorine) and assistance in dispersion (18).

Growth inside amoeba is considered a fundamental process in the life cycle of *L. pneumophila*. Although this bacterium is able to replicate in biofilm outside the host, this extracellular replication represents a minor contribution to maintain its populations in the environment (19, 20). The life cycle of *L. pneumophila* was primarily described as a biphasic cycle combining an intracellular replicative form and an extracellular infective form ready to infect new hosts (14). Nonetheless, currently the cycle is understood as a complex network in which the host interacts with several different developmental forms of the bacterium, such as exponential and stationary phase forms (EPF, SPF), mature infectious forms (MIFs),

filamentous and VBNCC (Viable But Non-Culturable cell) forms, among others (21). Regardless of the type of host, after invasion, *L. pneumophila* evades being transported to the lysosome and stablishing a unique endoplasmic reticulum-derived compartment where it is protected from degradation, known as *Legionella* containing vacuole (LCV). To remodel this compartment, the bacterium makes use of its major essential virulence factor, the Dot/Icm type IVB translocation system, which allows the translocation of effectors into the host-cell cytosol. The translocated effectors are responsible of manipulating the host cell functions (e.g. disrupting vesicle transport) and reprogramming the endosomal-lysosomal degradation pathway of the host to ensure its survival. Within the protected vacuole, *L. pneumophila* replicates until nutrients become limited. Nutrient limitation then leads to transition to the infectious forms, which will be ready to infect a new host after being released (22, 23).

1.3 Typing methods for *L. pneumophila*

Bacterial typing is an essential public health tool important for disease surveillance and outbreak investigation, for determining relatedness between bacterial strains and understanding pathogen transmission and evolution. The fundamental concept of bacterial typing is the ability to differentiate bacterial strains based on their phenotypes and genotypes. Classical typing methods focused on phenotypic characteristics, such as serotypes and nutrient demands. Molecular typing methods based on bacterial genotypes have increased and improved in the last decades, leading to enhanced methods for strain classification, assessment of clonal relatedness, detection of virulence genes, and drug resistance markers, as well as for the study of genetic evolution over time across geographic regions.

For almost 30 years the 16S ribosomal RNA gene (24) has provided a general framework for molecular studies of taxonomic relationships and the identification of bacterial diversity. However, despite its achievement in defining higher taxa, the 16S ribosomal RNA classification system lacks the resolution required to distinguish among closely related bacteria (25). The need for higher resolution characterization of isolates has led to the development of a wide range of strain typing methods (26), such as multilocus sequence typing (MLST), which has become a popular method for typing many organisms (27, 28).

MLST is used to study bacterial populations by investigating multiple housekeeping gene loci along the bacterial genome. MLST is based in the comparison of alleles instead of nucleotide sequences. In allele-based comparisons, each allelic change is considered a unique genetic event, independently of the number of nucleotide polymorphisms involved. Thus, direct comparison of alleles allow to study bacterial diversification combining vertical and horizontal genetic transfer events (29). Usually, most of MLST schemes are composed of seven MLST loci, for which each unique sequence is assigned to an arbitrary allelic number. The allelic

numbers of each locus are grouped together into an allelic profile or sequence type (ST), which is also designated using a numerical code. Sequence types are used to designate bacterial strains and can be grouped into clonal complexes or lineages to improve the understanding of the development of the bacterial population structure. The use of MLST housekeeping genes, however, does not provide enough discrimination for all typing purposes, including tracing back outbreaks or resolving differences among variants of single-clones. More recently, the advances in the sequencing technologies have facilitated whole-genome sequencing (WGS) of a wide variety of bacterial species (30). Several approaches have been used to detect variation among bacterial samples using WGS, for example, the analysis of single nucleotide polymorphisms, which has been successfully used in evolutionary and epidemiological studies of closely related and single-clone pathogens (31). Alternatively, variation can be also measured using a gene-by-gene approach, similar to MLST schemes (28).

In the case of *L. pneumophila*, several typing methods have been applied to subgroup and classified this bacterium. Monoclonal antibody (MAb) subgrouping and Sequence Based Typing (SBT) are the most common typing methods used in routine and reference clinical laboratories to distinguish between environmental and clinical strains during epidemiological investigations to identify the possible common environmental source. Monoclonal antibody subgrouping is a phenotypic method related to the lipopolysaccharide (LPS) characteristics of *L. pneumophila* (32, 33). Although the index of discrimination is low in comparison to other molecular typing methods, MAb is an ideal screening tool due to its low price and its ease of performance. SBT is considered the *gold standard* typing method for *L. pneumophila.* It is a molecular typing technique based in the sequencing of seven virulence-associated genes (*flaA, pilE, asd, mip, mompS, proA* and *neuA*) (34, 35). The combination of alleles is summarized as sequence type, as in the case of MLST. STs are allelic profiles that include the ordered string of allele numbers. In contrast to MLST, SBT uses genes subjected to selective pressures instead of housekeeping genes so it has a high index of discrimination. SBT can be applied directly as a culture-independent method in clinical samples when no isolates are available (36). Other molecular typing methods applied in the characterization of *L. pneumophila* strains are Pulse-field Gel Electrophoresis (PFGE), Amplified Fragment Length Polymorphism (ALFP) and a more recently described spoligotyping tool based on the diversity of the CRISPR (Cluster Regularly Interspaced Palindromic Repeats) locus for the subgrouping of the *L. pneumophila* serogroup 1 ST1 (37). While CRISPR spoligotyping is a complementary genotyping tool, PFGE and ALFP have been extensively used as main method for genotyping of *L. pneumophila.* (38–40). PFGE and ALFP are based in the digestion of genomic DNA with restriction enzymes and comparison of the resulting fragment patterns. Although both techniques possess high index of discrimination and can be applied not only to *L. pneumophila* but also to other *Legionella* species, they have the disadvantages of difficult intra- and inter-

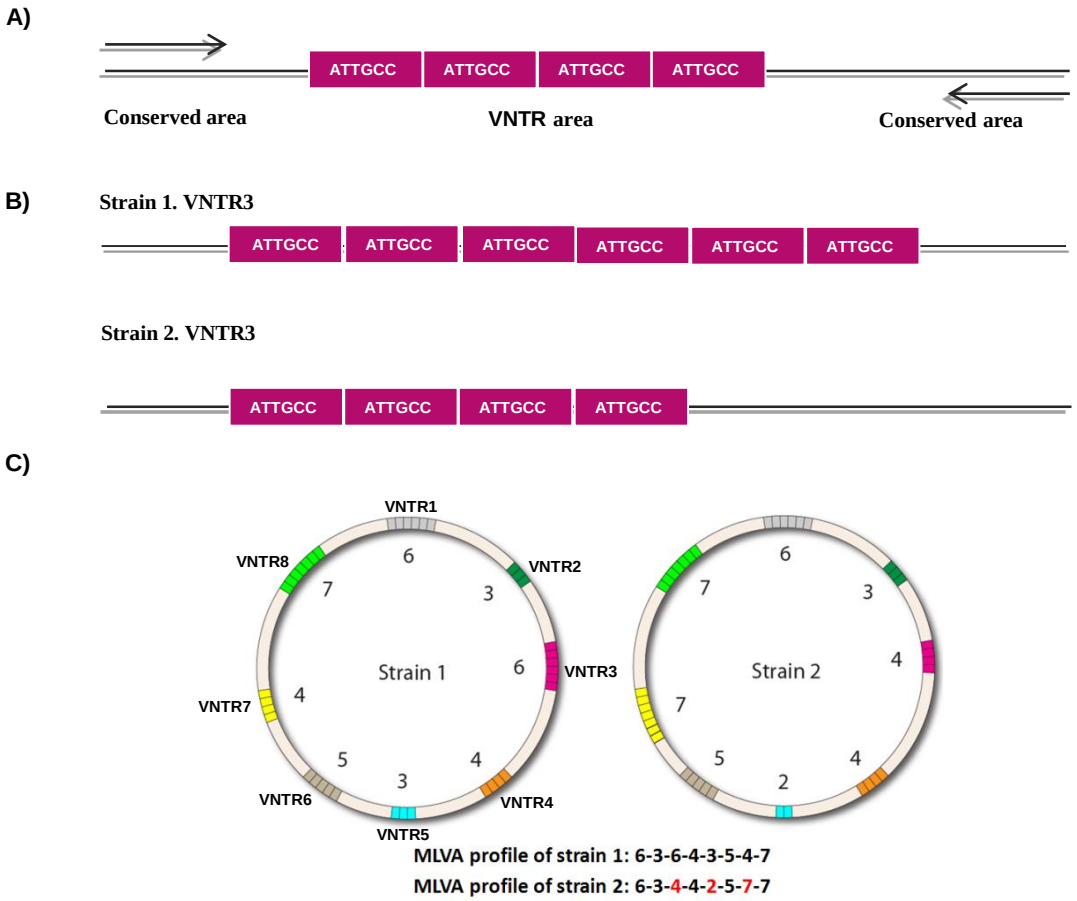
laboratory standardization and have been gradually replaced by SBT. In the last years, the great improvements in sequencing technologies have made the application of Whole Genome Sequencing (WGS) possible to study retrospective LD outbreaks and the structure of *L. pneumophila* populations (31, 41, 42). WGS has shown to have greater resolution compared to the traditional genotyping techniques described above and has demonstrated the feasibility of this technique for outbreak investigations.Therefore, WGS has the potential to replace current diagnostic and molecular epidemiological tools due to its multiple advantages (43, 44).

1.3.1 Multi Locus Variable number of tandem repeats (VNTR) Analysis (MLVA)

Multi locus variable number of tandem repeats analysis (MLVA) has emerged more recently as a method for genotyping foodborne bacterial pathogens and has been adopted for the typing of *L. pneumophila* (45–47). This method is based on the variation in the number of tandem repeated DNA sequences found in many different loci in the genome of microorganisms. DNA replication errors, such as slipped-strand mispairing, generate diversity in the number of tandem repeats observed between the strains. This method determines the number of tandem repeats, or copy units, at multiple variable number of tandem repeat (VNTR) loci along the genome. Normally, multiplex PCR amplification of the repeat and flanking regions is followed by amplicon sizing using capillary electrophoresis. The number of repeats units, or allele numbers, at each locus is calculated from the measured amplicon size. The string of alleles from multiple loci generates the MLVA profile, which is specific of each strain.

High resolution is the main advantage of MLVA genotyping, followed by reduced typing time, cost and throughput. These features make it suitable for outbreak investigations and surveillance of *L. pneumophila* (48) as well as for population studies (47, 49). Moreover, MLVA has been applied directly to DNA obtained from drinking water (46). MLVA can be applied as a complementary method to SBT, due to the increased resolution and the high concordance with the gold standard. Furthermore, studies comparing MLVA and WGS techniques carried out in other bacteria, such as *Escherichia coli* or *Clostridium difficile*, have revealed a high correlation between both genotyping methods, despite that MLVA only analyses a small part of the bacterial genome (50, 51). The analysis of MLVA genotypes through the construction of phylogenetic Minimum Spanning Trees (MSTs) allows inferring genetic relationships between the isolates and understanding their clonal relatedness (52). In addition to the application of genotyping methods to understand the clonal structure of *L. pneumophila* populations it is also important for the searching of possible ecological features responsible for the distinction of the different clonal complexes or lineages. Correlations between genetic clusters and ecology has been identified in other bacteria, such as species of the genus *Vibrio* (53, 54). The clonal complexes identified by MLVA can provide the basis for further ecological research.

Figure 2. **A**) Typical organization of a variable number of tandem repeat (VNTR) locus. The arrows point to the annealing sites for polymerase chain reaction (PCR) primers in the conserved region flanking the repeats. **B**) Example of the variation of the number of repeats in a particular VNTR locus (VNTR3) in two different strains (Strain 1 and 2). **C**) Variation in the number of repeats in eight VNTR (VNTR1 to VNTR8) loci along the genome of the two strains. The different number of repeats in the loci (numbers inside the genomes) is reflected in the MLVA profiles, which are specific for each strain.



1.4 Molecular epidemiology and infectivity of *L. pneumophila*

Epidemiological typing data collected from multiple studies have shown that most cases of LD have been caused by a small subset of serogroup 1 *L. pneumophila* strains. As previously mentioned, *L. pneumophila* serogroup 1 is the responsible of 70-90% of culture-confirmed cases of LD in Europe and the United States (6, 2). However, the proportions of serogroup 1 in clinical isolates do not really reflect the environment. In the natural environment, the diversity of serogroups is higher and serogroup 1 is less frequently isolated (55). Likewise, the prevalence of monoclonal subgroup MAb 1/3 positive or MAb2, which are serogroup 1 strains characterized by presenting a virulence-associated epitope, is very high in clinical strains but less frequent in the environment (56). As it occurs with serogroup 1 strains, there is little

overlap between clinical and environmental sequence types (STs) and common clinical STs are rarely isolated from the environment. In the study by Harrison et al. (2009) (55), where clinical and environmental populations of *L. pneumophila* in England and Wales were compared, it was reported that 98% of clinical isolates were serogroup 1; 92% were MAb 3/1 positive; and ST47, ST37, and ST62 accounted for 46% of all clinical isolates. Of the environmental isolates, only 56% were serogroup 1, 8% were MAb 3/1 positive, and 34% were ST1 or ST79. In addition, of all sequence types (STs) described by SBT, only a few of them appear usually responsible of most cases of LD. In England and Wales were predominantly ST47, ST37 and ST62, while in Unites States ST1, ST35, ST36, ST37, and ST222 were responsible for most of the cases of both outbreak-associated and sporadic cases (57). Several authors have suggested that clinical and environmental isolates cannot be distinguished on the basis of coding gene sequences (58). The same predominant clinical STs in England and Wales have been found to be common in other European countries, such as France (59). Despite that some of the most common strains appear restricted to specific regions, other are widespread distributed and have been found in very different geographic areas, e.g. ST1. Population studies applying MLVA genotyping have also revealed the presence of the same genotypes in very different environments and climatic areas, as Northern Europe and the Middle East (47, 49). At clonal level, the largest MLVA clonal complex (VACC) described in France, VACC1, which includes the pathogenic strain *L. pneumophila* Paris, was as well the predominant clonal complex observed in Israel. The broad distribution of clones suggests that geographical barriers would not be relevant for the development of the clones. Instead, adaptation to specific ecological niches could influence their distribution (60). Further research would be needed to better understand what ecological factors drive the adaptation of global and local clones to specific niches.

Although some *in-vitro* studies, using both their natural hosts and human macrophages, have shown the enhanced infectivity and persistence of serogroup 1 isolates in comparison to other serogroups (61), there is not a clear explanation for the different distribution of serogroups and STs in environmental and clinical populations. Besides the effect of the LPS, the virulence of *L. pneumophila* is mainly triggered by the activity of the Dot/Icm Type IVB secretion system. As indicated before, this system delivers approximately 300 effector proteins into the host cell, which manipulate the normal host cell activity in order to allow bacterial growth (62). Differences in pathogenicity between strains may be due to the differential functions of the different effectors. Therefore, determining the function of the multiple translocated proteins is essential to understand the virulence of *L. pneumophila.* However, high redundancy in the effectors has been reported so that the deletion of single proteins does not impair the replication of the bacterium inside the host (63). Standard genetic mutation analyses were unable to identify the function of many of these proteins in intracellular growth because the manipulation of multiple pathways by the bacterium masks defects resulting from loss of a single pathway. In

addition to the Dot/Icm Type IVB secretion system, *L. pneumophila* utilizes a type II secretion system, which also delivers proteins inside the host (64). It has been reported that mutants lacking the type II secretion system are defective of growth in amoeba, macrophages and mice, indicating that these proteins play an important role in the infectivity of the strains (65). The involvement of different secretion systems and the functional effector redundancy could be a strategy to preserve the ability to replicate in a wide host range.

1.5 Effect of temperature in the ecology of *L. pneumophila.*

Temperature represents a critical environmental factor that can mediate changes in growth, development, and pathogenesis of bacteria. In the case of *L. pneumophila,* temperature plays an essential role in its ecology. Temperature is crucial for the growth and abundance of this bacterium in natural and man-made freshwater systems. *L.pneumophila* are mesophilic bacteria, able to survive in a wide range of temperatures (20°C to 55°C), having their optimum growth temperature between 20°C to 40°C (66, 67). Due to the enhanced growth at warm temperatures, warm water systems (showers, air conditioners or cooling towers) have had always a high relevance in public health measures controlling *Legionella* infections. Outbreaks of LD have often been linked to contaminated freshwater systems. Therefore, heating the water above 50°C is a highly recommended disinfection measure to decrease the risk of outbreaks. However, several studies have observed that high temperatures (between 50°C and 60°C) can be associated with *Legionella* spp and *L. pneumophila* colonization. Kusnetsov et al. (1996) (68) demonstrated that the growth in liquid medium of all tested strains of *L. pneumophila* decreased at temperatures above 44-45°C, although the growth-limiting temperature was between 48.4°C and 50°C. Despite the decrease and the stop of cell multiplication, the strains produced carbon dioxide up to 51.6°C, indicating that the bacteria retained their metabolic activity even beyond the maximum temperature for cell multiplication. A recent study (69) has shown by molecular techniques, that the abundance of *Legionella* species and, in particular *L. pneumophila,* in a local freshwater system increased above 50°C. The increase of temperature from 49.2°C to 57.9°C correlated positively with the increase of *Legionella* cells per ml of water. Furthermore, Farhat et al. (2012) (70) reported in a pilot-scale hot water distribution system that *Legionella* could persist and recolonize the biofilm of the system after multiple rounds of heat shock at 70°C for 30 min. Heat shocks used to disinfect water systems usually entail the transformation of the cells into a VBNC (Viable But Non Culturable) state. VBNC is a physiological state in which bacteria cannot grow on standard growth media but retain certain features of viable cells, such as membrane integrity, metabolic activity or virulence. The physiological significance of the VBNC state is mostly thought to be an adaptive response to favour the long term survival under adverse environmental conditions (71). Cells in VBNC state can produce virulence

proteins (72). However, they only become infectious after its resuscitation on their natural hosts (73). On the other hand, in order to avoid the growth of *L. pneumophila* in cold water systems, the recommended temperature for storage and distribution of cold water is ideally below 20°C. Temperature does not only affect the multiplication of *L. pneumophila* in the environment but also affects its virulence (74, 75). In both studies it was demonstrated that strains grown at high temperatures (37°C and 41°C) were significantly more infective to guinea pigs than strains which were had been grown at lower temperatures (25°C).

1.6 Research approach

Understanding the clonal structure of population by using genotyping methods is necessary not only to identify the source of infection in case of outbreaks but also to understand overall the epidemiology of *L. pneumophila* and to assist the development of public health control strategies. Moreover, population studies covering distinct geographic areas help to identify global and local clones, which is essential for the establishment of molecular surveillance. The study of the physiological response of strains of these clones to environmental factors, as important as the temperature, is essential in order to understand their distribution in the environment and to get insights into their ecology. Finally, the assessment of the infectivity using human cells is crucial to evaluate the relevance of the clones for human health.

In this study, four main points were addressed. First, a biogeography of clinical and environmental strains in Germany; second, a biogeography and comparison of clinical and environmental strains between the two different climatic regions Germany and Middle East; third, an analysis of a representative subset of strains to characterize the temperature adaptation of relevant genotypes from Germany and the Middle East, and fourth, to assess the infectivity of relevant genotypes from Germany and Middle East.

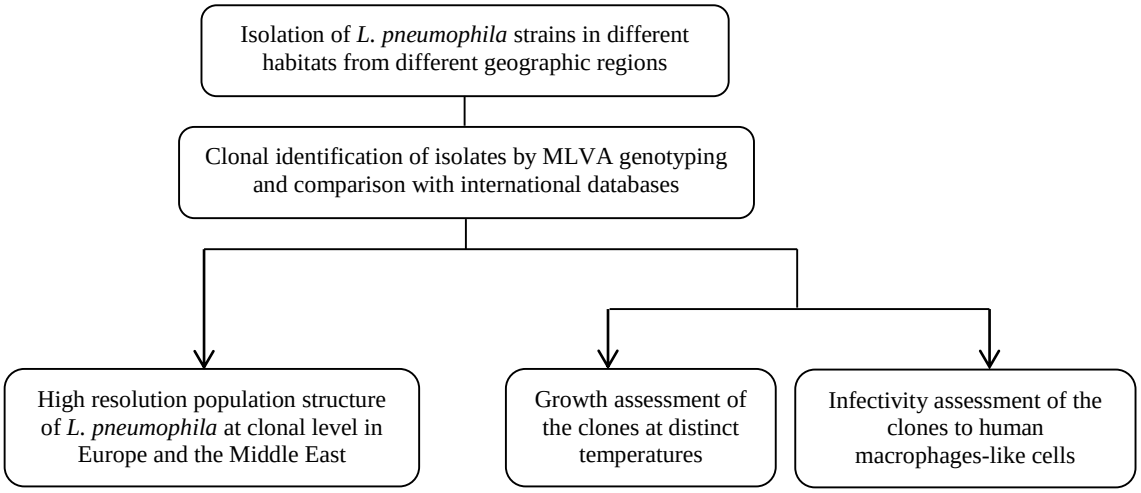
The MLVA genotyping method was selected due to its highly discriminatory power and performance to type environmental and clinical strains isolated from different habitats (biofilm, bulk water, cooling tower water) and different geographical regions (central Europe and the Middle East). The MLVA genotyping profiles obtained from isolates of these areas were compared with an international database (http://bacterial-genotyping.igmors.u-psud.fr/ Legionella2006) in order assess the biogeography of major genotypes and the respective clonal complexes. The major strains were physiologically characterized by studying their growth at a wide range of temperatures, and special interest was given to infer growth differences between clinical and environmental isolates. Finally, the infectivity of the clones to human macrophages was studied through different *in vitro* approaches.

1.6.1 Objectives of the thesis

Three objectives were defined in order to address the overall goal of the thesis, which is to better understand the biology of environmental and clinical isolates of *L. pneumophila* by the study of their clonal population structure in different environments using high resolution genotyping techniques, their growth behaviour in relation to the temperature and their interaction with human host cells. Therefore, the following objectives were approached:

- Chapter II: to establish and validate Multi Locus Variable number of tandem repeats Analysis (MLVA) genotyping method by comparing with the gold standard Sequence Based Typing (SBT) genotyping and to examine the genetic diversity and population structure of environmental and clinical *L. pneumophila* isolates in Germany by both techniques.
- Chapter III: to study the population structure and the distribution of genotypes of *L. pneumophila* in the Middle East, and its relation with the populations from Germany and the rest of Europe by comparing to the international *L. pneumophila* MLVA database.
- Chapter IV: to study the effect of the temperature in the growth of clinical and environmental isolates as well as of the most relevant clones. And finally, to assess the pathogenicity of the clones to human cells.

Figure 3. Project scheme of the current study. Molecular and physiological analyses were carried out to a large dataset of *L pneumophila* strains isolated in Europe and the Middle East. After the characterization of the isolates by MLVA genotyping, the growth at different temperatures was studied in order to investigate the role of this parameter in the ecology of the genotypes. Finally, in vitro assays were used to evaluated the infectivity potential of the main genotypes to human macrophages.



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Chapter II

Molecular diversity of clinical and environmental isolates of *L. pneumophila* across Germany based on Sequence Based Typing and Multi Locus Variable number of tandem repeats (VNTR) analysis (MLVA)

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2.1 Abstract

Typing methods are essential for the study of the distribution of clinical and environmental *L. pneumophila*, which is necessary in order to establish control measures and investigate the sources of outbreaks. In this study, Multi Locus Variable number of tandem repeats Analysis (MLVA) was established as a high resolution genotyping method and validated by comparing to Sequence Based Typing (SBT), the standard method for typing of *L. pneumophila.* Different MLVA schemes using eight (MLVA-8), 12 (MLVA-12) and 13 (MLVA-8(12)) VNTR loci were used. MLVA and SBT were applied to evaluate the genetic diversity of 311 environmental and clinical *L. pneumophila* isolates for different parts of Germany. All different MLVA schemes presented high reproducibility and typeability. In general, the discriminatory power of three MLVA schemes was higher than that of SBT. In MLVA genotyping, the resolution improved when increasing the number of VNTR studied. Therefore, MLVA-8(12) scheme showed the highest resolution. The majority of isolates (70%) were identified as single genotypes. However, certain genotypes, i.e. Gt4(17) and other closely related strains differing in a single locus, were frequently observed. These genotypes corresponded to worldwide distributed sequence types, such as ST1 and ST23, and were found across different regions of Germany. Little overlap was detected between environmental and clinical isolates and the same genotypes, as Gt4(17), were dominant in both groups of isolates. Clonal analysis of the strains of *L. pneumophila* in Germany indicated that the population structure of *L. pneumophila* isolated from Germany resulted from the combined action of widespread clonal complexes and genotypes, i.e. clonal complex VACC1 and Gt4(17), along with genetic differentiation at shorter geographic distances.

2.2 Introduction

The incidence of Legionnaires´ disease (LD) has been increasing during the last years in Germany (1, 2). Most of the cases that contributed to the increase were attributed to infections in the community, with a minor number of travel-associated and nosocomial cases. Additionally, major outbreaks have occurred since 2010 in Warstein, Ulm and Zweibrücken (3–5).

In cases of LD, it is crucial to detect the source of the infection as promptly as possible by comparing clinical and environmental strains of *L. pneumophila* to apply decontamination measures to control and prevent further cases. With this purpose, and in general, for the study of the genetic diversity and distribution of *L. pneumophila* strains, which could be important for public health control strategies, numerous genotypic typing methods have been applied to the typing of *L. pneumophila* in the last years. These methods include amplified fragment length polymorphism (AFLP), pulsed-field gel electrophoresis (PFGE) (6), sequence-based typing (SBT) (7, 8) or more recently whole genome mapping (WGM) (9) and whole genome sequencing (WGS) (10). PFGE and SBT have been widely used but SBT is still considered the current gold-standard assay for epidemiological investigations, due to its high typeability, interlaboratory reproducibility and epidemiologic concordance (11). Moreover, the European Society for Clinical Microbiology Study Group on Legionella Infections (ESGLI) maintains an active and continually updated allele database. In a study where *L. pneumophila* phylogenies were compared by SBT and WGS approaches, it was shown that STs could reflect precisely the whole genome genotypes (12). However, WGS remains to be challenging to substitute SBT as routine and outbreak investigation genotyping due to high costs and lack of standadised sequence analysis.

Multilocus variable number of tandem repeats (VNTR) analysis (MLVA) has been largely used to type different pathogens (13–15). VNTRs consist of relatively short DNA fragments repeated in tandem that can vary in copy number among strains. MLVA assays have exhibited high level of discrimination among strains and reduced typing time and costs. Several studies have used MLVA for the genotyping of *L. pneumophila* strains (16–18). They showed the high correspondence between MLVA genotypes and sequence types (STs) and an important increase of the resolution when applying MLVA, which is relevant for understanding clonal populations. Due to its advantages, MLVA could be used to complement SBT and gain insights in the clonal structure population of *L. pneumophila* as well as to help selecting strains for further whole genome sequencing.

The populations of *L. pneumophila* isolates in Germany have been previously studied by SBT (19, 20). However, so far no such studies using MLVA or WGS have arised. The aim of this study was to investigate the clonal population structure of *L. pneumophila* in Germany by using MLVA genotyping. To this purpose, two MLVA typing assays previously proposed for *L.*

pneumophila strains were optimized and validated and a total of 311 environmental and clinical isolates from across Germany were MLVA genotyped. The MLVA genotypes were compared with those genotypes from the MLVA *Legionella* database containing strains from Europe and multiple reference strains. MLVA genotypes were compared to SBT and aspects of diversity, geographical distribution and differences of clinical and environmental isolates were assessed. The results obtained in this study have been used as a base to select genotypes for whole genome sequencing.

2.3 Material and methods

2.3.1 Isolation of *Legionella* spp. and strain collection

Water and biofilm samples were taken monthly between June 2013 and July 2014 from a cooling tower located at the HZI campus (E building) as part of a routine sampling plan. During the same period, hot water samples were taken from a shower in the men toilets (D0.21). Moreover, biofilm samples were taken from two faucets of two kitchens of the HZI (D2 building) that were replaced on June 28th 2013.

Isolation of *Legionella* from water samples was performed by culture using a modification of the International Standard method ISO 11731:1998 **(**International Organization for Standardization, 1998**)**, based on a filtration procedure and cultivation of bacteria on selective media. 300 ml of water collected from the sump of the cooling tower, a chamber where water flows to facilitate pump suction, were concentrated by filtration on a sterile 47 mm, 0.2 µm pore size, polycarbonate membrane (Whatmann Nucleopore, UK). After filtration, bacteria collected on the membrane were resuspended in 15 ml of sterile water and vortexed for 10 min. 1:10 dilutions of the concentrate were made and three plates of GVPC (Glycine-Vancomycin-Polymyxin- Cycloheximide medium, Oxoid Thermoscientific, Germany) were plated with 1 ml and 0.1 ml of 1:10 concentrate, respectively. In the case of hot water, two liters were filtered as described above and bacteria resuspended in 10 ml of sterile water. 1 ml and 0.1 ml of a 10-fold dilution of this concentrated water were plated onto three plates of BCYE (Buffered Charcoal Yeast Extract Agar, Oxoid Thermoscientific, Germany). Plates were incubated for 7 to 10 days at 37°C under aerobic conditions and humidified atmosphere. Round, white, purple or greenish colonies with smooth edges and with the centre usually brighter than the borders, were suspected to be *Legionella* spp. and were counted as positive and subcultured in BCYE and BCYE without L-cysteine to be confirmed. The isolated colonies growing only on BCYE agar but not on BCYE without L-cysteine were considered to be *Legionella* colonies according to ISO 11731:1998. Five colonies of each plate were selected when possible. Isolates were kept in YEB (Yeast Extract Broth) supplemented with 10% glycerol at -80°C.

Biofilm samples were taken in the cooling tower from the bottom and the walls of the chamber containing the drift eliminators when the surfaces were moist by using nylon swabs (FLOQSwabs, Copan Flock Technologies, Italy). Swaps were suspended in 1 ml of sterile water and vortex gently. 0.1 ml was plated in three GVPC plates and 0.1 ml of 1:10 diluted suspension was plated in three BCYE plates. The incubation, examination of the plates and preparation of isolates were done as described above for the water samples.

A total of 283 *L. pneumophila* strains from the period 1998-2013 were kindly provided by the National Reference Laboratory for Legionella Infections in Dresden, Germany (**Table A1**). 81 strains were received by post in an insulated storage container as living biomass plated in BCYE plates. Isolates kept at -80°C as described above. From the rest of the strains, DNA was received in 1.5 ml screw cap Eppendorf tubes, which was kept at -20°C until later analysis.

2.3.2 Identification of *Legionella* spp. isolates

Bacterial DNA was extracted using the DNeasy-Kit (Qiagen; Hilden, Germany). Briefly, four to five colonies were suspended in phosphate buffer saline (PBS) and centrifuged for 10 min at 7500 rpm (Heraeus Biofuge, Germany). The pellet was suspended in 180 µl of ATL lysis buffer from the kit and 20 µl of Proteinase K and was incubated at 56°C for 30 min. 200 µl of AL-Buffer and 200 µl absolute ethanol were added to the mix and all volume was transferred to a spin column. After 1 min centrifugation at 8000 rpm, the columns were washed with 500 µl AW1 and AW2 buffer. DNA was eluted in 50µl of AE elution buffer after a final centrifugation step of 1 min at maximum speed and finally quantified by Nanodrop spectrophotometer (NanoDrop, Thermo Scientific, Germany).

PCR amplifications of parts of the bacterial 16S rRNA gene were performed to test at the molecular level the genus and the species of the isolates. A *Legionella* genus-specific PCR was carried out using the primers 17F (5´-GGCCTACCAAGGCGACGATCG-3´) and 28R (5´-CACCGGAAATTCCACTACCCTCTC-3´) (21). PCR was done using 50 ng of DNA of the isolates in a final volume of 25 µl, starting with an initial denaturation for 15 min at 95°C. A total of 35 cycles (30 sec at 95°C, 30 sec at 66.5°C, and 30 sec at 72°C) was followed by a final elongation for 10 min at 72°C. A *L. pneumophila* species-specific PCR was performed using the primers Lp-16S_246-248F (5´-CCTGGGCTTAACCTGGGAC-3´) and Lp-16S_246-248R (5´-CTTAGAGTCCCCACCATCACAT-3´) (21) and 50 ng of DNA in a final volume of 25 µl. An initial denaturing step of 15 min at 95°C was followed by 35 cycles (45 min at 95°C, 45 min at 60°C and 45 sec at 72°C) and a final elongation step of 10 min at 72°C. 1.5 and 1 U of HotStarTaq DNA polymerase were used respectively (Qiagen, Hilden, Germany). Isolates were further characterized by complete sequencing of the 16S rRNA gene. To amplify the complete gene (about 1500 bp) a PCR was carried out using the primers 27F (5´-AGAGTTTGATCMTGGCTCAG-3´) and 1492R (5´-TACGG YTACCTTGTTACGACTT -3´)

(21). 50 ng of DNA were amplified in a total volume of 50 µl by a denaturing step of 15 min at 95°C, followed by 30 cycles (60 sec at 95°C, 40 sec at 56°C and 60 sec at 72°C) and an elongation step of 10 min at 72°C. 3.75 U of HotStarTaq DNA polymerase was used for the amplification. PCR products were purified using MinElute PCR Purification kit (Qiagen, Hildem, Germany) and purified amplicons were amplified in a sequencing reaction (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems, UK) using an additional primer 1087R (5´-CTCGTTGCGGGACTTAACCC -3´) to full cover the sequence of the 16S rRNA gene. Products were purified again using MinElute PCR Purification kit (Qiagen, Hildem, Germany) following manufacturer instructions and analyzed by Sanger sequencing using capillary electrophoresis with fluorescence detection (ABI Prism 3100 Genetic Analyzer).

The serogroup of *L. pneumophila* isolates obtained at the HZI campus were identified using a latex agglutination test (Oxoid, UK) following manufacter´s instructions. This test is specific for the especies *L. pneumophila* and allowed a separated identification of *L. pneumophila* serogroup 1 and serogroups 2-14. All isolates were subjected to the test, however, other non-pneumophila *Legionella* species were expected to be negative in the agglutination reaction. Some of the isolates (n=24) were subjected to monoclonal subgrouping using the Dresden panel (22, 23). Information regarding serogroups, monoclonal subgroups and sequence types was provided for the isolates obtained from the Reference Laboratory in Dresden.

2.3.3 Multi Locus Variable number of tandem repeats (VNTR) Analysis (MLVA) of *L. pneumophila* isolates

Two MLVA protocols were previously published for *L. pneumophila* (16, 18). One protocol comprised eight VNTR marker genes (MLVA-8) and the second one was composed of 12 VNTR genes (MLVA-12). The MLVA-8 typing scheme carried out in this study consisted of two multiplex PCR (multiplex 1, multiplex 2) using the set of primers previously described (16, 21) (**Table S1**). In each multiplex reaction, four VNTR loci were amplified. 25 µl PCR reactions contained one to two ng of DNA template, 1x Multiplex PCR Master Mix (Qiagen, Hilden, Germany) and 1.25 pmol of each fluorescently labelled primer (VIC-, NED-, FAM- and NET- labelled forward primers from Applied Biosystems, Foster City, CA). The PCR program comprised an initial denaturation step of 95 ºC for 15 minutes, 35 cycles of 30 seconds at 94 ºC, 90 seconds at 60 ºC and 60 seconds at 72ºC, followed by a final elongation step for 30 minutes at 60 ºC.

The MLVA-12 scheme for typing *L. pneumophila* strains consisted of an optimized multiplex touchdown PCR where 12 VNTRs markers were amplified using the set of primers previously described (18) (**Table S1**). Amplification was carried out by using 5 to 10 ng of

template DNA in a final volume of 25 µl, comprising 1x Multiplex PCR Master Mix (Qiagen, Hilden, Germany) and 1.25 pmol of each primer. Forward primers were labelled with VIC, PET, 6-carboxyfluorescein (FAM), and NED dyes (Applied Bio- systems, Foster City, CA) (Sobral et al 2011). PCR comprised an initial denaturation cycle for 15 min at 95°C followed by a first phase of 15 cycles (30 sec at 95°C, 120 sec at 78°C with a decrease in this step of 1.2°C in every cycle, and 70 sec at 72°C), a second phase of 15 cycles (30 sec at 95°C, 120 sec at 60°C and 70 sec at 72°C, with an increase of 5 sec per cycle in this last step) and a final step of 15 min at 68°C. PCR products of both protocols were checked in 1.5% agarose-gel electrophoresis and later purified on filter columns (MSB HTS PCRapace/C (96) kit, Stratec Molecular, Germany) according to the manufacturer instructions. Purified PCR products were diluted 1:10 with molecular grade water (Roth, Germany) and 1µl of the dilution was added to 8.8µl of highly deionized formamide (Applied Biosystems, UK) and 0.2µl of GeneScan 1200 LIZ Size Standard (Applied Biosystems, USA). The mixture was denaturated for 3 min at 95°C in a thermoblock, cooled down on ice for at least 1 min and spun down briefly in a Multifuge 1 centrifuge (Heraeus, Germany). VNTRs were analyzed by fluorescent capillary electrophoresis using a 3730xL sequencer (Applied Biosystems) with pre-run voltage of 8.0 kV, run voltage of 8kV, injection voltage of 1.8kV and injection time of 15 sec.

Both typing schemes were stablished in our laboratory, however, MLVA-12 protocol, less time-consuming and providing more information than MLVA-8, was primarily used for the typing of *L. pneumophila* isolates. To obtain additionally the MLVA-8 scheme and further create a combination of both MLVA typing schemes, MLVA-12 multiplex PCR was carried out as described and Lpms17, the only locus contained in the protocol MLVA-8 but missing in MLVA-12, was amplified separately in a singleplex PCR. The singleplex PCR was performed by using 1 to 2 ng of DNA in a final volume of 50 µl, 6 pmol of primer (forward primer fluorescent labelled) and 1.5 U of HotStarTaq DNA polymerase (Quiagen). Each dNTP and $MgCl_2$ was added in a concentration of 0.1 µM and 2 µM, respectively. Amplification was achieved by 15 min denaturation at 95°C, followed by 35 cycles (30 sec at 95°C, 30 sec at 62°C and 30 sec at 72°C) and a final step of 10 min at 72°C. Products purification and capillary electrophoresis was done as described above for MLVA-12. Additionally, all 13 VNTR markers were sequenced (forward primers non-fluorescent labelled) for *L. pneumophila* Philadelphia-1 reference strain by cycle sequencing (ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit; Applied Biosystems) as a quality control measure.

2.3.4 Data analysis

Sequences analyses were carried out using the software Sequencer version 5.2.4 (Genecodes, USA). Capillary electrophoresis data were analysed using GeneMapper software (version 3.7, Applied Biosystems) to perform sizing and to calculate the number of repeats in

the PCR fragments. Each locus was identified according to colour. Data Fragment sizes were converted into repeat numbers based on the formula:

number of repeats (bp) = fragment size (bp) − flanking regions (bp) /repeat size (bp).

A null allele ("0") was assigned when no amplicon was detected. The number of repeats was rounded to the nearest integer value. An allele number string (MLVA profile), based on the number of repeats at each locus, was assigned to all isolates. An arbitrary numerical code was assigned to each MLVA-8 and MLVA-12 genotype. To designate MLVA-8(12) genotypes (containing all 13 VNTR) a joint code, including the code in MLVA-8 and in MLVA-12, was used. Identical profiles were named with the same code and null alleles were taken into account when defining the profiles. Suggestions for optimized panels of VNTRs were determined using the Automated Selection of Typing Target Subsets (AuSeTTS) Analysis (24).

To quantitatively compare and evaluate the congruence among different typing methods (MLVA-8, MLVA-12 and SBT), Simpson's Index of Diversity, Adjusted Rand and Wallace coefficients were calculated using the online tool provided on http://darwin.phyloviz.net/ComparingPartitions. To measure the variation of the number of repeats at each VNTR locus, the Hunter-Gaston Discrimination Index (HGDI), which is a modification of the Simpson's Index of Diversity, was calculated using http://www.hpa-bioinfotools.org.uk/cgi-bin/DICI/DICI.pl. Data with the calculated number of repeats were imported into Bionumerics (version 5.0, Applied Maths) and the unweighted pair group method with the arithmetic mean (UPGMA) clustering method was applied using the categorical coefficient and a cut-off value of 60% similarity was applied to define clusters. MLVA-8 allelic profiles available at the international *Legionella* database (http://bacterial-genotyping.igmors.u-psud.fr/ Legionella2006) were downloaded and imported to Bionumerics for comparison. Minimum spanning trees using categorical coeficients and scaling with member number were also produced in Bionumerics. Fisher's exact test was performed to check statistical significance (GraphPad Prism, v. 5.0, USA).

2.4 Results

2.4.1 Isolation and identification of *Legionella* spp. isolates

The ISO 11731-2:2004 isolation protocol was initially selected and used for the *Legionella* isolation from the hot water on a shower in the men toilets (room D0.21) and the cooling tower water at the HZI campus. However, isolation was not successful using this method and no isolates could be obtained from any of the two systems. By using a modification of the ISO 11731:1998 standard protocol, a total of 74 *Legionella* isolates were obtained during June 2013 to July 2014 (**Table S2**). 66 isolates were obtained from the cooling tower, 61 were isolated from the cooling tower water and 5 from biofilm. Only 3 *Legionella* isolates could be

obtained from hot water in April 2014 and five isolates were obtained from the dismantled faucets (June 2013), from the cooling tower water and 5 from biofilm. Isolation was not possible during the summer months of 2013 and in June 2014, probably due to an increase in the biocide concentration applied to prevent *Legionella* growth in the cooling tower during the warm months.

Analysis of the 16S rRNA gene showed that 53 isolates matched strains *L. pneumophila,* as Philadelphia-1, Paris, Corby, Lorraine or Los Angeles. 21 isolates showed a negative result in the *L. pneumophila* species-specific PCR as well as in the latex agglutination reaction for serogroup 1 and serogroups 2-14. According to the 16S rRNA analysis these isolates corresponded to the species *L. longbeacheae*.

2.4.2 Validation of MLVA genotyping assays

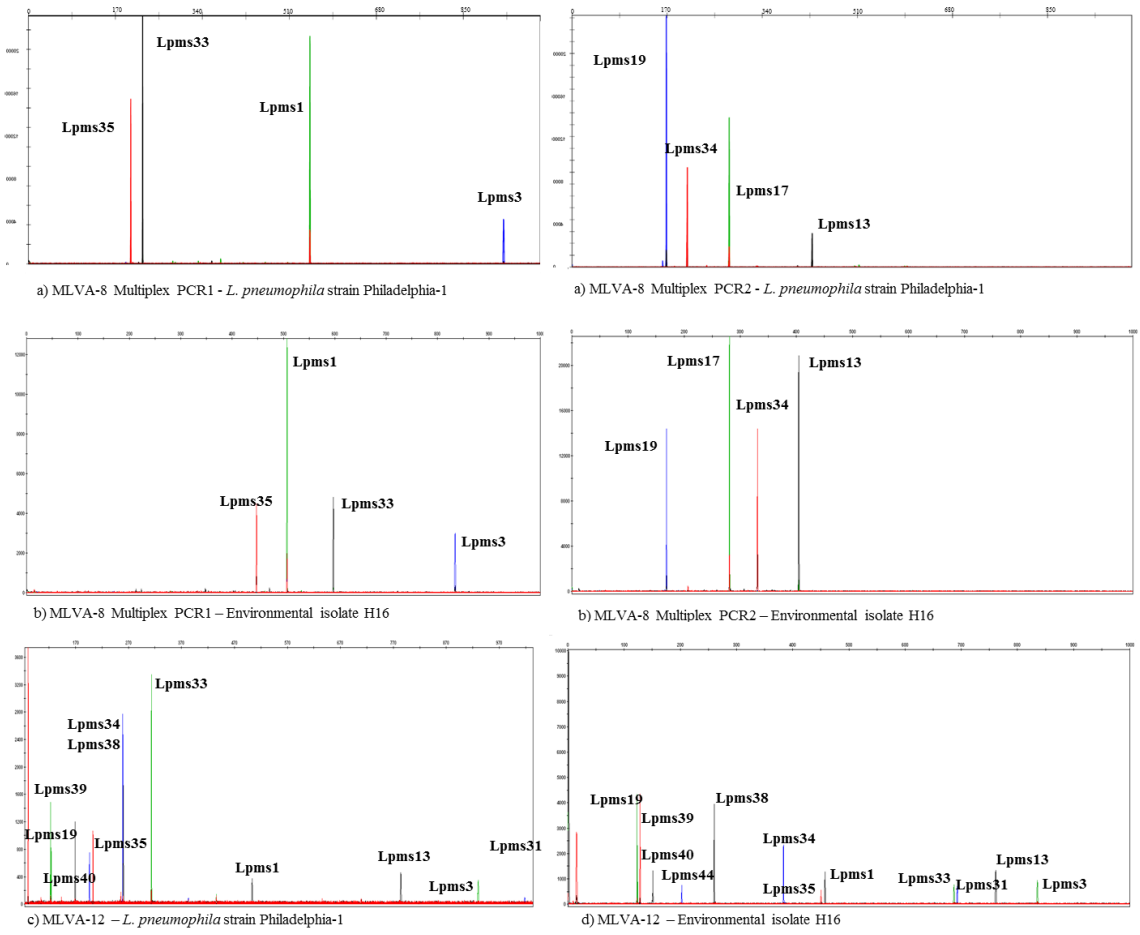
The robustness and performance of the MLVA assays described for *L. penumophila* has already been demonstrated when it was initially established (16, 18). Nevertheless, the modified protocols were validated in our laboratory as recommended in standard guidelines for development and application of MLVA methods (25, 26). For validation, a set composed of 36 environmental and clinical isolates (outbreak-associated and sporadic isolates), as well as reference strains, was used.

The stability of the VNTRs markers along passages in vivo and in vitro systems was already shown to be very high (S=1.00) in both MLVA-8 and MLVA-12 typing schemes and was therefore, not tested again in our laboratory. MLVA markers were originally selected based on their specificity for *L. pneumophila* strains (27), nonetheless, this was assessed additionally for a larger set of *Legionella* species (*L. anisa* ATCC 35292T, *L. feeleii* ATCC 35072T, *L. jordanis* ATCC 33623T, *L. hackeliae* ATCC 35250T, *L. bozemanii* ATCC 33217T, *L. erythra* ATCC 35303T, *L. longbeache* ATCC 33462T, *L. micdadei* ATCC 33218T , *L. oakridgensis* ATCC 33761T and *L. wadsworthii* ATCC 33877T). None of the loci were amplified in the multiplex PCR for any of the *Legionella* species tested. The absence of PCR products was confirmed by agarose-gel and by capillary electrophoresis, whose sensitivity is greater, and no peaks could be detected (**Figure 1**).

The reproducibility of MLVA-8 scheme was tested by comparing the profiles obtained for three *L. pneumophila* reference strains (*L.pn.* Philadelphia-1, *L.pn.* Paris and *L.pn.* Lens) with their published profiles (16) (**Table S3**). For the MLVA-12 scheme, the reproducibility was confirmed by comparison with the previously published profile of *L.pn.* Philadelphia-1 (18). Furthermore, the 13 alleles of *L.pn.* Philadelphia-1 were sequenced in order to verify the copy number and the accuracy of size determination by capillary electrophoresis (CE). The sequencing of the markers showed sizes comparable to the sizes expected and same number of repeats and, consequently, same profile. However, a difference of few base pairs for all VNTRs

was observed. This difference was especially larger for Lpms31 (8 bps). The same phenomenon was observed among the expected or theoretical sizes and the actual sizes of the amplicons obtained using CE. Both sizes differed in a few base pairs and for most of the alleles the sizes determined by CE were smaller than the theoretical size. Size differences between CE and sequencing have already been shown in other studies (28–30). The differences in the number of base pairs increased with the size of the amplicon, regardless of the dye (VIC®-, NED®-, FAM- or NET) used for labelling. The maximum difference observed was 21 bp (Lpms31). In this case, this difference of size affected the number of repeats, which decreased from 17 to 16.

Figure 1. Representative electropherograms of the MLVA-8 and MLVA-12 PCR products separated by CE and identified according to their sizes and colors. Electropherograms correspond to MLVA-8 PCR products of panel 1 and panel 2 of **a**) *L. pneumophila* Philadelphia-1 and **b**) the environmental isolate from the cooling tower H16; MLVA-12 PCR products of **c**) *L. pneumophila* Philadelphia-1 and **d**) *L. pneumophila* isolate H16.



The repeatability of PCRs (MLVA-12 multiplex and Lpms17-singleplex PCR) and the size measurement of the alleles by capillary electrophoresis were validated by typing twice a panel of 36 non-related and epidemiologically related isolates (panel I). The results showed a complete agreement of the deduced repeat number at all loci (**Table S4**). Panel l was besides

used to assess typeability. Results showed 100% typeability, since all isolates tested were able to be assigned to a MLVA type. The typeability of some loci (Lpms1, Lpms19, Lpms38, Lpms40) where null alleles (no fragments were amplified by PCR) were observed, was confirmed using singleplex PCR reactions in order to rule out suboptimal multiplex conditions as a cause for amplification failure. Singleplex PCR showed no amplification for any of the loci so PCR failure was rejected. The presence of null alleles could be considered to be sequence mismatches in the primer binding region.

2.4.3 Individual VNTR marker evaluation

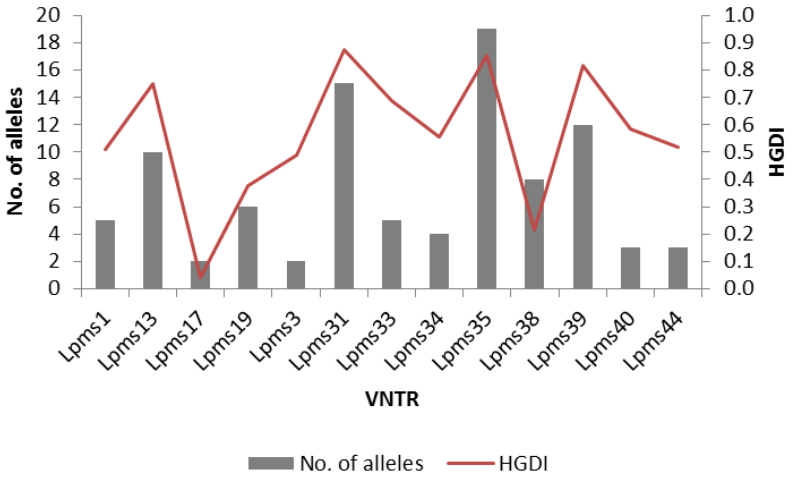
The VNTR markers used in both typing schemes were analyzed individually using a larger set of 149 isolates (**Table S5**). This group of 149 isolates was comprised of 73 MLVA-8(12) genotypes (D=0.964, 95% CI 0.949-0.980). The Hunter Gaston diversity index was calculated for each of the 13 VNTR markers (**Table 1**). Four VNTRs were considerably more discriminative than the rest: Lpms13, Lpms31, Lpms35 and Lpms39 (**Figure 2**). These markers could discriminate more than 10 alleles, being Lpms35 the most variable one (19 alleles). Three of them were minisatellites (Lpms13, Lpms31, Lpms35) and only one a microsatellite (Lpms39). Eight markers showed occurrence of null alleles, due to non-amplification of the loci during PCR. The markers presenting a higher percentage of null alleles were the four microsatellites (6 to 8 bp units) and Lpms19, one of the VNTR with smallest repeat unit size (21 bp).

Table 1. Characteristics of the 13 VNTR markers analyzed in panel II based on 149 isolates.

VNTR designation	Repeat unit size (bp)	No. of alleles	% of null alleles	HGDI[1]	CI[2] (95%)
Lpms1	45	5	0	0.508	(0.450-0.567)
Lpms13	24	10	1.34	0.748	(0.692-0.803)
Lpms17	39	2	0	0.04	(1.000-0.084)
Lpms19	21	6	7.38	0.375	(0.282-0.467)
Lpms3	96	2	0	0.487	(0.458-0.516)
Lpms31	45	15	1.34	0.873	(0.855-0.892)
Lpms33	125	5	0	0.689	(0.648-0.729)
Lpms34	125	4	0.67	0.555	(0.501-0.609)
Lpms35	18	19	0	0.853	(0.817-0.889)
Lpms38	8	8	3.36	0.214	(0.124-0.304)
Lpms39	6	12	6.71	0.815	(0.782-0.849)
Lpms40	6	3	18.12	0.584	(0.527-0.641)
Lpms44	6	3	16.78	0.519	(0.444-0.593)

[1]Hunter Gaston diversity index, [2] Confidence interval of HGDI.

Figure 2. Representation of the number of alleles and the Hunter Gaston diversity index (HGDI) index of the 13 VNTR markers.



Both, the presence of null alleles, and the characterization of these four markers as the most discriminative VNTRs, was already shown (18). However, the 13 VNTR markers were evaluated by the software AuSeTTS (Automated Selection of Typing Target Subsets) to determine the combination of loci that maximizes the discriminatory power (**Table 2**).

Table 2. Combination of VNTRs markers that maximize discriminatory power

No. of VNTR	ID (95% CI)[1]	No. of genotypes	VNTR Targets
0	0	1	
1	0.873 (0.855-0.892)	15	Lpms31
2	0.944 (0.928-0.96)	36	Lpms13, Lpms31
3	0.955 (0.94-0.97)	52	Lpms13, Lpms19, Lpms31
4	0.959 (0.944-0.974)	58	Lpms13, Lpms19, Lpms31, Lpms38
5	0.961 (0.946-0.977)	65	Lpms13, Lpms19, Lpms31, Lpms35, Lpms38
6	0.962 (0.948-0.978)	66	Lpms13, Lpms19, Lpms31, Lpms33, Lpms35, Lpms38
7	0.963 (0.949-0.979)	69	Lpms13, Lpms19, Lpms31, Lpms33, Lpms35, Lpms38, Lpms40
8	0.964 (0.949-0.979)	72	Lpms13, Lpms19, Lpms31, Lpms33, Lpms35, Lpms38, Lpms39, Lpms40
9	0.964 (0.949-0.98)	73	Lpms13, Lpms17, Lpms19, Lpms31, Lpms33, Lpms35, Lpms38, Lpms39, Lpms40
10	0.964 (0.949-0.98)	73	Lpms1, Lpms13, Lpms17, Lpms19, Lpms31, Lpms33, Lpms35, Lpms38, Lpms39, Lpms40
11	0.964 (0.949-0.98)	73	Lpms1, Lpms3, Lpms13, Lpms17, Lpms19, Lpms31, Lpms33, Lpms35, Lpms38, Lpms39, Lpms40
12	0.964 (0.949-0.98)	73	Lpms1, Lpms3, Lpms13, Lpms17, Lpms19, Lpms31, Lpms33, Lpms34, Lpms35, Lpms38, Lpms39, Lpms40
13	0.964 (0.949-0.98)	73	Lpms1, Lpms3, Lpms13, Lpms17, Lpms19, Lpms31, Lpms33, Lpms34, Lpms35, Lpms38, Lpms39, Lpms40, Lpms44

[1]Simpson Index of Diversity and Confidence Interval

A combination using a total of 9 markers would be able to discriminate the same number of genotypes that were obtained using the 13 markers. This optimal combination of markers would be formed by the four most discriminative markers mentioned above and other group of 5 markers that include mini- and microsatellites (Lpms17, Lpms19, Lpms33, Lpms38 and Lpms40). Interesting, by using a subset of 6 markers (Lpms13, Lpms19, Lpms31, Lpms33, Lpms35, Lpms38) would allow to differentiate 90% of the genotypes. According to the results by AuSeTTS, markers Lpms3, Lpms13, Lpms34 and Lpms38 could be excluded. The use of a protocol containing less number of VNTR but same efficiency could reduce the price of the typing and facilitate the optimization of the multiplex PCR.

2.4.4 Comparison of Multi Locus Variable number of tandem repeats Analysis (MLVA) and Sequence Based Typing (SBT)

A panel of 149 isolates (panel II, **Table S5**), comprising 112 clinical and 37 environmental isolates with known STs and different epidemiological background, was used to test the discriminatory power of the different MLVA schemes and to compare with the "gold standard" SBT as recommended in the guidelines for application of MLVA methods. Overall, the resolution showed by MLVA-12 (ID=0.964, 95% CI 0.948-0.979) scheme was greater than the resolution showed by MLVA-8 (ID=0.948, 95% CI 0.929-0.966) (**Table 3**).

Table 3. Simpson's Index of Diversity of the different typing methods

Typing method	No. of genotypes	Simpson's ID[1]	CI[2] (95%)
ST	45	0.931	(0.910-0.953)
MLVA-8	50	0.948	(0.929-0.966)
MLVA-12	71	0.964	(0.948-0.979)
MLVA-8(12)	73	0.964	(0.949-0.980)

[1] Index of Diversity, [2] Confidence interval

Twenty-one genotypes more were obtained when a higher number of loci was used. Nevertheless, discrimination was in both cases equal or higher than 0.95, which is the thereshold generally used to consider a typing method as "ideal" (25). In addition, both MLVA schemes showed higher discrimination than SBT (ID=0.931, 95% CI 0.910-0.953), which could discriminate only 45 genotypes out of the 149 isolates in contrast to 50 genotypes obtained by MLVA-8 and 71 by MLVA-12. This higher discrimination of MLVA in comparison to SBT is consistent with previous results (16, 18, 31). The combination of 13 loci using the 12 loci included MLVA-12 plus Lpms17, the only locus present in the MLVA-8 but not in the MLVA-12 scheme, was generated and designated using a joint nomenclature as MLVA-8(12). Yet, only

two genotypes more were found when using in addition Lpms17 (ID=0.964, 95% CI 0.949-0.980). Therefore, the discriminatory power of this new combination did not differ considerably of the MLVA-12 resolution.

The congruence between MLVA and STB was quantified using the adjusted Wallace coefficient (32) (**Table 4**). This coefficient indicates the probability that a pair of isolates which are assigned to the same type by one typing method are also typed as identical by the other method. A good directional correlation between MLVA and SBT results was observed: the probability of two isolates having the same MLVA-8(12) type also sharing the same SBT type (ST) was 86.9%. This probability was smaller for MLVA-12 (84.8%) and for MLVA-8 (76.4%). By contrast, the possibility that two isolates sharing the same ST also shared the same MLVA type was 57.3% for MLVA-8, 43.6% for MLVA-12 and 43.6% for MLVA-8(12). These differences can be explained by the higher discriminatory power of MLVA compared to SBT.

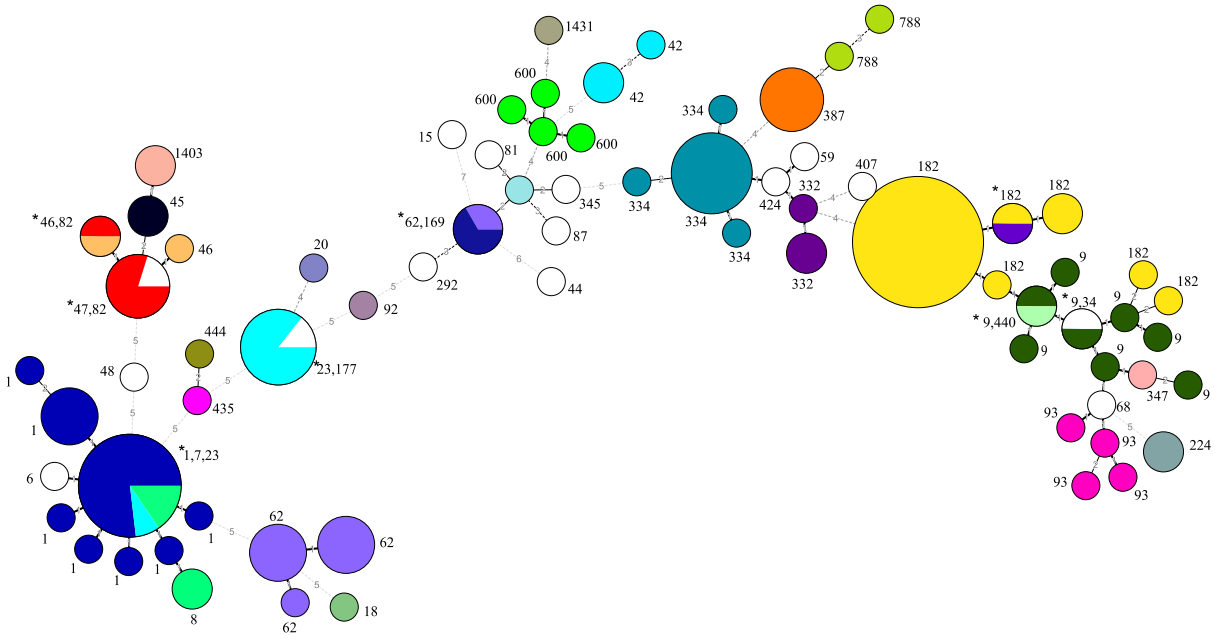
Table 4. Congruence between typing methods expressed by adjusted Wallace coefficients and analytical 95% confidence interval.

Typing method	**ST**	**MLVA-8**	**MLVA-12**	**MLVA-8(12)**
ST		0.573 (0.454-0.692)	0.436 (0.308-0.563)	0.436 (0.309-0.564)
MLVA-8	0.764 (0.657-0.870)[1]		0.669 (0.530-0.808)	0.669 (0.531-0.808)
MLVA-12	0.848 (0.771-0.924)	0.976 (0.945-1.000)		0.977 (0.946-1.000)
MLVA-8(12)	0.869 (0.798-0.940)	1.000 (1.000-1.000)	1.000 (1.000-1.000)	

[1] Confidence interval

A minimum spanning tree of the MLVA-8(12) data, grouped by SBT using colors (**Figure 3**), shows the typing resolution of both methods and how clustering with MLVA correctly aggregates, in most of the cases, isolates with similar sequence types (ST). In general, MLVA-8(12) allowed further differentiation of isolates with identical STs, although was also possible to find some cases where a MLVA-8(12) type corresponded to 2 or up to 3 distinct STs. An example of it is ST1, which can be subtyped into 8 different MLVA-8(12) types (indicated by * in Figure 3). On the contrary, one of MLVA-8(12) type does not match exclusively with ST1 but also with ST7 and ST23. This could occur due to the presence of recombination, a genetic mechanism that has been shown to occur in *L. pneumophila* (33).

Figure 3. Minimum spanning tree of 149 *L. pneumophila* isolates typed by MLVA and SBT. Each circle represents a MLVA-8(12) type; the size of the circle is scaled according to the number of isolates within a given MLVA-8(12) type. The numbers in the different lines connecting the MLVA-8(12) types represent the VNTRs variants. Each color represents an ST and STs appear numbered. Asterisks denote MLVA-8(12) genotypes matching more than one ST. Due to the large number of STs, some of the MLVA-8(12) types containing only a single strain are left white.



2.4.5 Phylogeography and population structure of *L. pneumophila* isolates in Germany using MLVA analysis

2.4.5.1 Diversity of *L. pneumophila* in Germany

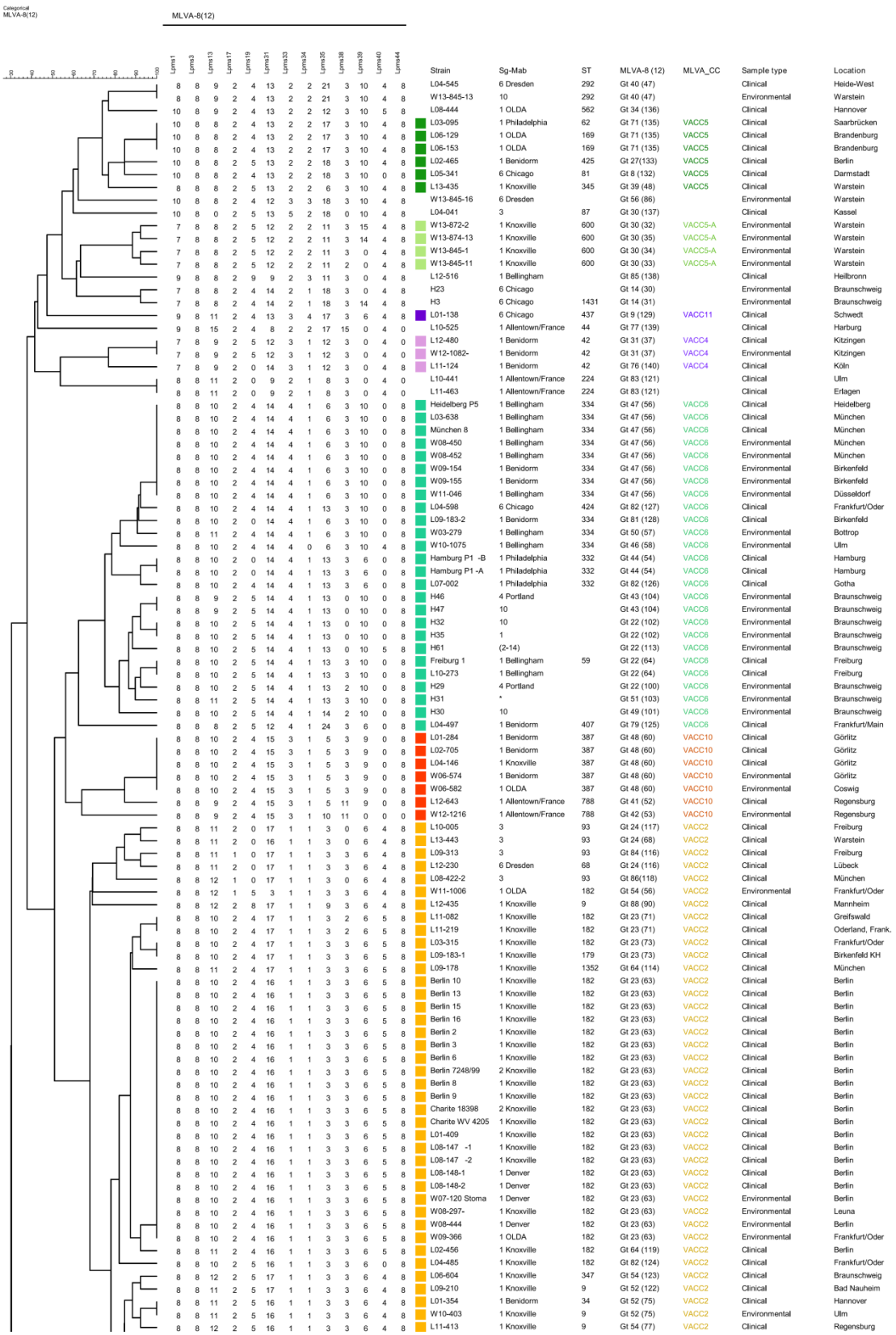
A total of 249 *L. pneumophila* strains from the period 1998-2013 was provided by the National Reference Laboratory for Legionella Infections in Dresden, Germany. Specifically, 147 strains were of clinical origin and 102 were from environmental sources. Environmental strains were isolated during routine surveillance programs for *Legionella* control as well as during outbreak investigations whilst clinical strains were isolated from patients that represented cases of nosocomial or community infections as well as outbreaks. Environmental and clinical strains related to cases of nosocomial or community infections were obtained from 57 cities across Germany spanning 16 regions. Strains that represented travel-associated cases of legionellosis (n=19) were as well provided, in addition to four reference strains and five strains for which important information, as their location, was missing. A total of 311 *L. pneumophila* isolates, collected at the HZI and obtained from the Legionella National Reference Laboratory, was MLVA-8(12) genotyped. However, a reduced group of isolates (n=179) was used for diversity and population structure analysis of *L. pneumophila* in Germany. Travel-associated isolates, reference strains and strains with missing information were excluded from the study.

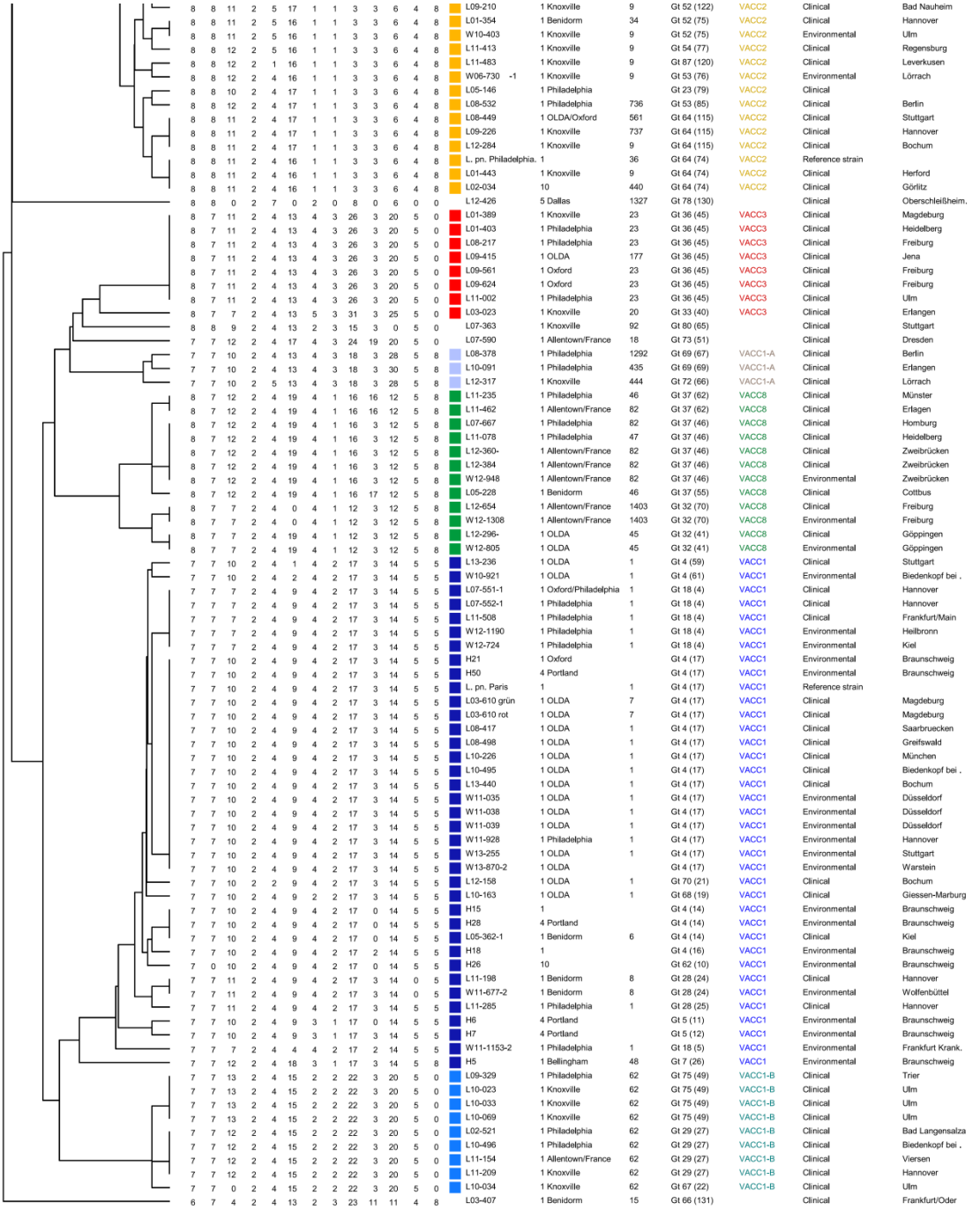
Furthermore, in order to avoid redundancy due to sample bias, the number of isolates obtained at the HZI (n=62) was reduced to the minimum number of genotypes found (n=20). Isolates obtained after the legionellosis outbreak occurred in Warstein, 2013 (n=71), were as well reduced to the minimum of genotypes (n=9) for the same reason.

The 179 isolates, 122 from clinical origin and 57 environmental isolates, were typed in 60 MLVA-8 genotypes (ID=0.954, 95% CI 0.939-0.969), 91 MLVA-12 genotypes (ID=0.972, 95% CI 0.96-0.984) and 92 MLVA-8(12) genotypes (ID=0.973, 95% CI 0.962-0.984) The 29% (n=27) of MLVA-8(12) genotypes were found at least twice, and the rest 71% (n=66) were singletons. About 30% of the isolates (n=51) belonged to only 4 genotypes. Among the most frequent isolated genotypes were Gt4(17) (n=16, 8.9%) and Gt36(45) (n=7, 3.9%), which belonged to ST1 (1,4,3,1,1,1,1) and ST23 (2,3,9,10,2,1,6), two worldwide highly abundant sequence types.

MLVA-8 genotypes were clustered together with the MLVA-8 genotypes of the *Legionella* MLVA database using UPGMA analysis and 60% similarity in order to define the groups as previously done (18, 31). Nine MLVA Clonal Complexes or VACCs (VACC1, VACC2, VACC3, VACC4, VACC5, VACC6, VACC8, VACC10 and VACC11), which were consistent with the clusters existing at the database, were formed (**Figure 4**). When using the combination of 13 loci of MLVA-8(12), adding the markers of MLVA-12 scheme (Lpms31, Lpms38, Lpms39, Lpms40, and Lpms44), few changes were observed in the clusters. Strain L07-590 was not included in VACC1, L08-378, L10-091 and L12-317 formed a group dissociated from VACC1 (VACC1-A) and another group of nine clinical isolates was dissociated from VACC1 and formed an independent cluster (VACC1-B). Strains L08-444, L10-525, W13-872-2, W13-874-13, W13-845-1 and W13-845-11 were dissociated from VACC5. The last four genotypes formed a separated group (VACC5-A). So, three new clonal complexes were observed with the addition of the MLVA-12 markers. In total, 92.2% of isolates (n=165) were clustered in 12 VACCs (**Figure 4**) and the rest 7.8% (n=14) were found as singletons. VACC2 was the largest cluster including 49 isolates (27.4%), followed by VACC1 (36 isolates, 20.0%) and VACC6 (26 isolates, 14.5%). The majority of strains from these clonal complexes were Sgp1 (88.9% in VACC1, 87.8% in VACC2, 69.2% in VACC6%). VACC1 and VACC2 were characterized by epidemic strains such as Paris and Philadelphia-1, respectively.

Figure 4. Categorical UPGMA analysis deduced from the clustering of 179 environmental and clinical *L. pneumophila* isolates from panel III using MLVA-8(12) genotyping. MLVA clonal complexes (VACC) of three or more genotypes are shown in different colors.



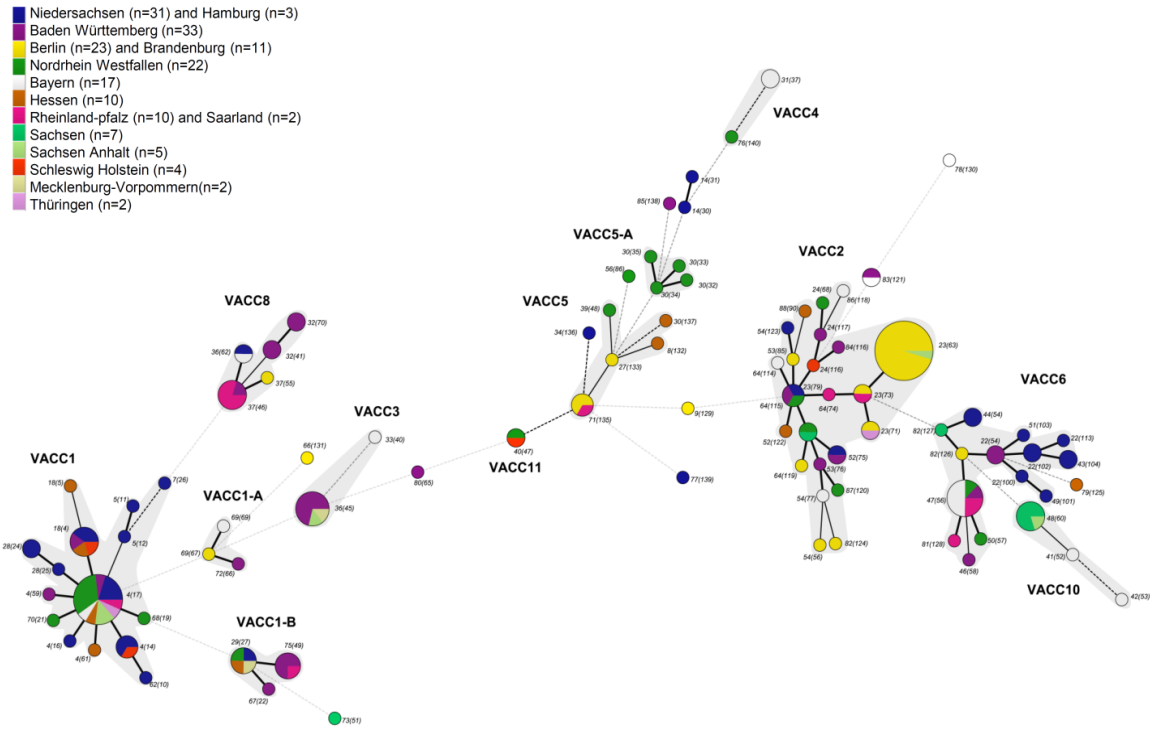


2.4.5.2 Geographical structure of *L. pneumophila* isolates in Germany

L. pneumophila isolates were obtained from all 16 federal states of Germany, although, the number of isolates was not even distributed across the states. Most isolates (n=124, 69.3%) were obtained from five states (Niedersachen, Baden Würtemberg, Berlin, Nordrhein-Westfalen and Bayern). Several genotypes showed a wide distribution across the states (**Figure 5, Figure 6A**), with genotype Gt4(17) (VACC1) being the most widespread profile (8/16 states). It corresponds to ST1, a well-known worldwide distributed genotype. In general, the geographical

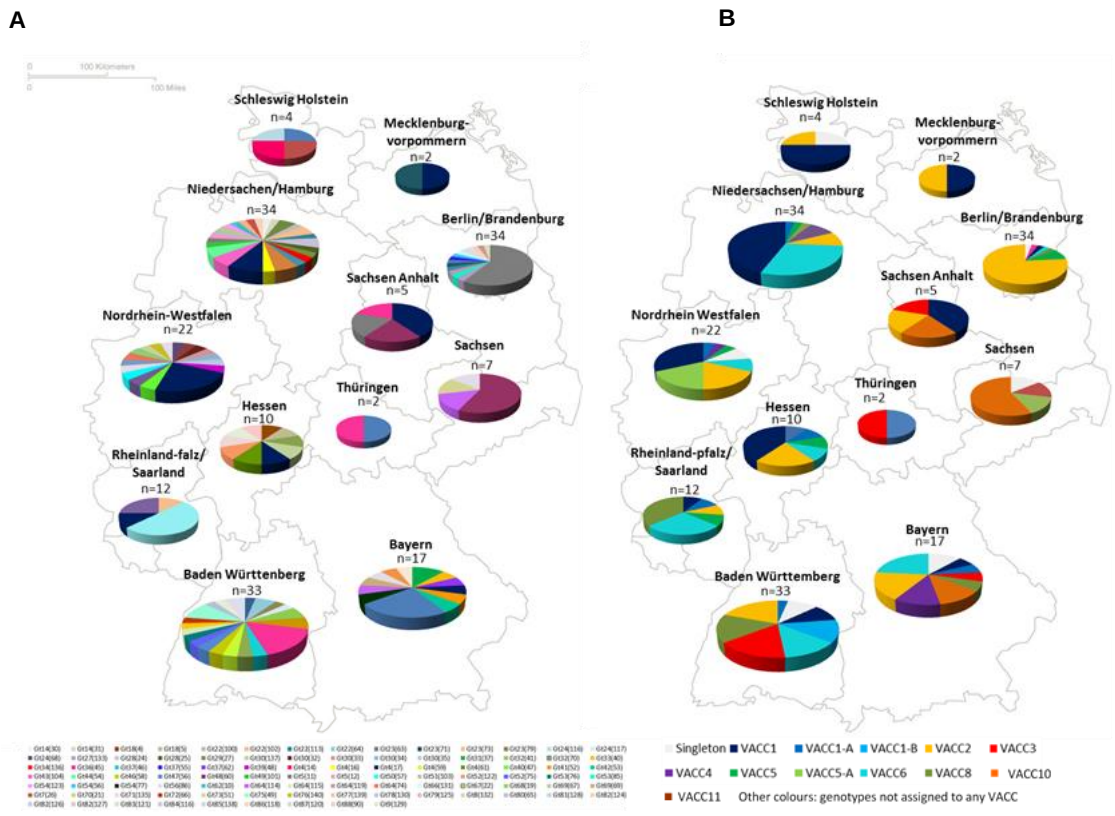
distribution of VACCs was varied (**Figure 5, Figure 6B**). The largest clonal complexes showed to be broadly distributed. In the case of VACC1 (ID=0.811, 95% CI 0.693-0.929), present in 10/16 states), the wide distribution was due to the wide spreading of its most abundant genotype Gt4(17). For VACC2 (ID=0.815, 95% CI 0.703-0.928, 12/16 states) and VACC6 (ID=0.902, 95% CI 0.809-0.994, 9/16 states) extensive distribution could be due to the higher genotype diversity. Only one small cluster, VACC5-A, formed by 4 isolates of 4 distinct genotypes, was exclusively found in the city of Warstein, during the legionellosis outbreak in August 2013.

Figure 5. Minimum spanning tree obtained with Bionumerics on the MLVA-8(12) profiles of 179 *L. pneumophila* strains. MLVA clonal complexes (VACC) appear shaded. Pie charts represent the 92 different MLVA-8(12) genotypes of Germany with size proportional to genotype frequencies and the different colours refer to the 16 different states of Germany (see legend). Thicknesses of the branches represent the number of different of loci.



To better evaluate the levels of genetic diversity in Germany, results from this study were compared to the variability in Europa reported to the *Legionella* MLVA database. Isolates clustered into the largest clonal complexes VACC1 and VACC2, were also reported in several countries across Europe (Greece, England, Sweden, Portugal, Spain or Denmark among other). VACC6 was reported in Sweden and France, where one of its genotypes was colonizing most parts of the hot water system in the French city of Rennes (18). Several VACC6 genotypes corresponded to ST332 (7,10,17,6,14,11,3) and ST424 (7,10,17,3,13,14,9), which were reported according to the *L. pneumophila* Sequence-Based Typing database only from Germany.

Figure 6. Geographical distributions of MLVA-8(12) genotypes (A) and clonal complexes (B) across Germany. Each colour represents a genotype or clonal complex (see legend).



2.4.4.3 Comparison of environmental and clinical isolates

Serogroup and, when possible, monoclonal antibody (MAb) subgroup of the clinical (n=122) and environmental (n=57) isolates are shown in **Table 5**. Overall, 90.2% of clinical isolates were sgp1 (serogroup 1) compared to 71.9% of environmental isolates ($P<0.05$). Sgp3 and sgp5 were exclusively found in clinical isolates (4.1% and 0.8%, respectively). Likewise, sgp4 was only found in environmental isolates (10.5%). All three serogroups (sgp3, sgp5 and sgp4) were found in cases of nosocomial and community acquired infections as in routine samplings in different countries according to the SBT *Legionella* database (3.5%, 1.8% and 0.9% of the isolates reported to the database).

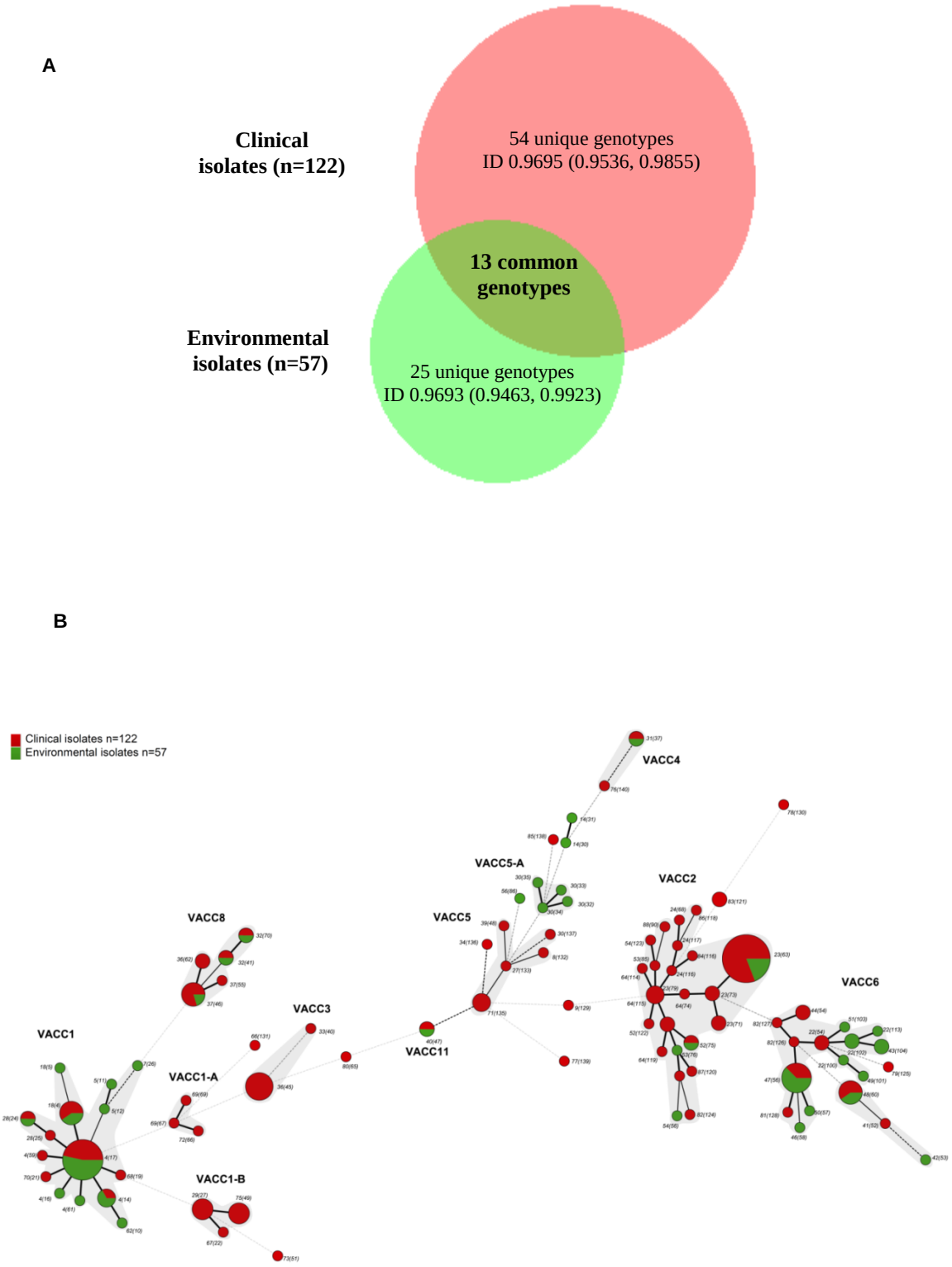
Likewise, according to the MAb serotyping, 68% of the clinical isolates were confirmed to be part of the virulence-associated 'MAb3/1 positive' subgroup [also known as MAb2+ ve' subgroup or "Pontiac" (34)], compared to 33.3% of the environmental isolates ($P<0.05$). Clinical isolates obtained from the three large *Legionella* outbreaks that have been occurred in the past years in Germany (Ulm, 2010; Zweibrücken, 2012 and Warstein, 2013) were typed as sgp1 MAb3/1 positive.

Table 5. Distribution of clinical isolates (n=122) and environmental isolates (n=57) of *L. pneumophila* by phenotype (serogroup and MAb subtype) in Germany.

Serogroup (sgp)	MAb subtype	Clinical isolates		Environmental isolates	
		No.	Frequency (%)	No.	Frequency (%)
1	Philadelphia	19	15.6	4	7.0
1	Allentown/France	11	9.0	3	5.3
1	Benidorm	12	9.8	5	8.8
1	Knoxville	41	33.6	7	12.3
	MAb 1/3 +ve	*83*	*68.0*	*19*	*33.3*
1	OLDA	15	12.3	10	17.5
1	Oxford	2	1.6	1	1.8
1	OLDA/Oxford	1	0.8	0	
1	Bellingham	6	4.9	6	10.5
1	Denver	2	1.6	2	3.5
	MAb 1/3 -ve	*26*	*21.3*	*19*	*33.3*
1	Oxford/Philadelphia	1	0.8	0	
1	Unknown	0		3	5.3
	Total sgp1	*110*	*90.2*	*41*	*71.9*
3		5	4.1	0	
4		0		6	10.5
5	Dallas	1	0.8	0	
6	Chicago	3	2.5	2	3.5
6	Dresden	2	1.6	1	1.8
10		1	0.8	5	8.8
(2-14)		0		2	3.5
	Total non-sgp1	*12*	*9.8*	*16*	*28.1*
Total		*122*	*100.0*	*57*	*100.0*

Table 6 shows the distribution of MLVA-8(12) genotypes and STs of clinical and environmental isolates. As shown before, 92 MLVA-8(12) genotypes were observed among the 179 isolates (ID=0.973, 95% CI 0.962-0.984). Among the 122 clinical isolates, 67 genotypes were identified (ID=0.970, 95% CI 0.954-0.985) while 38 genotypes were identified among the 57 environmental isolates (ID=0.969, 95% CI 0.946-0.992). Only 13 genotypes were common to the two groups (**Figure 6A**), 54 genotypes were exclusively found among clinical isolates and 25 among environmental isolates. In both groups, the distribution frequency of the genotypes was skewed (**Table 6**). The common genotypes were the most abundant ones in each group (frequency of >5%), in particular, two genotypes, Gt4(17) and Gt23(63), accounted for 20.5% and 21% of clinical and environmental isolates, respectively. The majority of genotypes in each group were found only once. Gt4(17), as already mentioned, corresponds to ST1.

Figure 6. **A**) Venn diagram of the number of MLVA-8(12) genotypes shared among clinical and environmental *L. pneumophila* isolates. **B**) Minimum spanning tree of the MLVA-8(12) profiles of 179 *L. pneumophila* strains. MLVA clonal complexes (VACC) appear shaded. Clinical isolates are represented in red and environmental isolates in green in both graphics.



ST1 is usually very frequent in the environment (36) but it can also be associated to nosocomial and community acquired infections as well ((37, 38). Gt23(63) corresponds to ST182, a sgp1 genotype isolated predominantly in Germany, particularly in the area of Berlin and Brandenburg responsible for multiple infections. Gt47(56), the third more frequent genotype among environmental isolates (8.8%), was also present among the clinical genotypes (2.5%). It matches ST334, a sequence type subtyped as MAb3/1 negative found primarily in the environment in Germany. **Figure 6B** shows the clonal relatedness between clinical and environmental isolates. Several clonal complexes were formed exclusively by clinical isolates (VACC1-A, VACC1-B, VACC5), while VACC5-A was composed only by environmental isolates. The rest of clonal complexes shared both types of isolates. VACC2, despite of containing environmental isolates was formed mostly by numerous clinical genotypes.

According to the epidemiology, most isolates were related to cases of LD (nosocomial and community-acquired infections). Only the isolates obtained from the cooling tower in the HZI campus were not considered to be related to any case of infection. No correlations were found between the MLVA 8(12) genotypes, serogroup or MAb subgroups and the origin of the isolates.

Table 6. Distribution of MLVA-8(12) genotypes among clinical (n=122) and environmental isolates (n=57) of *L. pneumophila*.

		Clinical isolates		**Environmental isolates**	
ST	**MLVA- 8(12)**	**No.**	**Frequency (%)**	**No.**	**Frequency (%)**
ST182	Gt 23(63)	17	13.9	4	7.0
ST1	Gt 4(17)	8	6.6	8	14.0
ST23/ST177	Gt 36(45)	7	5.7	0	
ST62	Gt 29(27)	4	3.3	0	
ST62	Gt 75(49)	4	3.3	0	
ST47/ST82	Gt 37(46)	4	3.3	1	1.8
ST9/ST561/ST737	Gt 64(115)	3	2.5	0	
ST62/ST169	Gt 71(135)	3	2.5	0	
ST387	Gt 48(60)	3	2.5	2	3.5
ST334	Gt 47(56)	3	2.5	5	8.8
ST1	Gt 18(4)	3	2.5	2	3.5
ST9/ST440	Gt 64(74)	2	1.6	0	
ST59	Gt 22(64)	2	1.6	0	
ST46/ST82	Gt 37(62)	2	1.6	0	
ST424	Gt 83(121)	2	1.6	0	
ST332	Gt 44(54)	2	1.6	0	
ST189	Gt 23(73)	2	1.6	0	

		Clinical isolates		Environmental isolates	
ST	**MLVA- 8(12)**	**No.**	**Frequency (%)**	**No.**	**Frequency (%)**
ST182	Gt 23(71)	2	1.6	0	
ST93	Gt 24(117)	1	0.8	0	
ST93	Gt 24(68)	1	0.8	0	
ST93	Gt 84(116)	1	0.8	0	
ST93	Gt 86(118)	1	0.8	0	
ST92	Gt 80(65)	1	0.8	0	
ST9	Gt 52(122)	1	0.8	0	
ST9	Gt 54(77)	1	0.8	0	
ST9	Gt 87(120)	1	0.8	0	
ST9	Gt 88(90)	1	0.8	0	
ST87	Gt 30(137)	1	0.8	0	
ST81	Gt 8(132)	1	0.8	0	
ST8	Gt 28(24)	1	0.8	1	1.8
ST788	Gt 41(52)	1	0.8	0	
ST736	Gt 53(85)	1	0.8	0	
ST68	Gt 24(116)	1	0.8	0	
ST62	Gt 67(22)	1	0.8	0	
ST562	Gt 34(136)	1	0.8	0	
ST46	Gt 37(55)	1	0.8	0	
ST45	Gt 32(41)	1	0.8	1	1.8
ST444	Gt 72(66)	1	0.8	0	
ST44	Gt 77(139)	1	0.8	0	
ST437	Gt 9(129)	1	0.8	0	
ST435	Gt 69(69)	1	0.8	0	
ST425	Gt 27(133)	1	0.8	0	
ST424	Gt 82(127)	1	0.8	0	
ST42	Gt 31(37)	1	0.8	1	1.8
ST42	Gt 76(140)	1	0.8	0	
ST407	Gt 79(125)	1	0.8	0	
ST347	Gt 54(123)	1	0.8	0	
ST345	Gt 39(48)	1	0.8	0	
ST34	Gt 52(75)	1	0.8	1	1.8
ST334	Gt 81(128)	1	0.8	0	
ST332	Gt 82(126)	1	0.8	0	
ST292	Gt 40(47)	1	0.8	1	1.8
ST20	Gt 33(40)	1	0.8	0	
ST182	Gt 64(119)	1	0.8	0	
ST182	Gt 82(124)	1	0.8	0	
ST18	Gt 73(51)	1	0.8	0	
ST15	Gt 66(131)	1	0.8	0	
ST1403	Gt 32(70)	1	0.8	1	1.8
ST1352	Gt 64(114)	1	0.8	0	

ST	MLVA- 8(12)	Clinical isolates		Environmental isolates	
		No.	Frequency (%)	No.	Frequency (%)
ST1327	Gt 78(130)	1	0.8	0	
ST1292	Gt 69(67)	1	0.8	0	
ST1	Gt 28(25)	1	0.8	0	
ST1	Gt 4(14)	1	0.8	2	3.5
ST1	Gt 4(59)	1	0.8	0	
ST1	Gt 68(19)	1	0.8	0	
ST1	Gt 70(21)	1	0.8	0	
	Gt 85(138)	1	0.8	0	
ST9	Gt 53(76)	0		1	1.8
ST788	Gt 42(53)	0		1	1.8
ST600	Gt 30(32)	0		1	1.8
ST600	Gt 30(33)	0		1	1.8
ST600	Gt 30(34)	0		1	1.8
ST600	Gt 30(35)	0		1	1.8
ST48	Gt 7 (26)	0		1	1.8
ST334	Gt 46(58)	0		1	1.8
ST334	Gt 50(57)	0		1	1.8
ST182	Gt 54(56)	0		1	1.8
ST1431	Gt 14(31)	0		1	1.8
ST1	Gt 18(5)	0		1	1.8
ST1	Gt 4(61)	0		1	1.8
	Gt 14(30)	0		1	1.8
	Gt 22(100)	0		1	1.8
	Gt 22(102)	0		2	3.5
	Gt 22(113)	0		1	1.8
	Gt 4(16)	0		1	1.8
	Gt 43(104)	0		2	3.5
	Gt 49(101)	0		1	1.8
	Gt 5(11)	0		1	1.8
	Gt 5(12)	0		1	1.8
	Gt 51(103)	0		1	1.8
	Gt 56(86)	0		1	1.8
	Gt 62(10)	0		1	1.8
Total		*122*	*100*	*57*	*100.0*

2.5 Discussion

2.5.1 Validation of MLVA assays

Germany is fourth in the EU with respect to the total cases of LD according to the last surveillance report of the European Center for Disease prevention and Control (39). Previous studies have suggested that the incidence of LD may increase under warm and wet

meteorological conditions, which could be due to the global climate change (40–42). Therefore, surveillance of environmental sources and proper maintenance of man-made freshwater systems are key in the prevention of legionellosis. Surveillance of *Legionella* in the environment is also indispensable to validate the efficacy of decontamination procedures, for risk assessment, when evaluating the potential transmission or amplification sources at a facility, and for tracking the sources of outbreaks. Cultivation is still considered the gold standard for detection of *Legionella* in the environment yet other non-culture methods are available, as serology or nucleic acid-based detection methods, especially qPCR (43). Cultivation can be inaccurate as a result of overgrowth by other microorganisms on the agar plates, can generate false positives (44) and can be ineffective due to the presence of viable but non culturable (VNBC) *Legionella* cells (45). However, cultivation allows obtaining isolates that can be identified and characterized phenotypically and genetically which is essential for epidemiological studies.

Several typing methods have been applied for subgrouping of *Legionella* species. In particular, most of them were applied for subtyping of *L. pneumophila* since this species is the responsible for more than 90% of the cases of LD. Among the typing methods, such as Pulse-Field Gel Electrophoresis (PFGE) or Amplified Fragment Length Polymorphism (ALFP), monoclonal antibody subtyping (MAb) and sequence-based typing (SBT) are the preferred epidemiological typing methods for comparison of clinical and environmental isolates. Monoclonal antibody subtyping is easy to perform and although its index of discrimination is not as high as for other methods, it is still useful to distinguish environmental isolates not related to the clinical isolates during outbreak investigations. SBT has high typeability, interlaboratory reproducibility and generally a high index of discrimination (46). However, some STs are very common (ST1) and when those STs are responsible for causing LD higher discrimination is needed in order to find out the source of the infection. (40, 47, 48). Therefore, the use of a combination of typing methods is recommended (49).

Multiple Locus Variable number of tandem repeats Analysis (MLVA) has emerged as a method for the subtyping of bacterial pathogens. Studies performing MLVA genotyping with other pathogens showed the increase of resolution of MLVA when it was compared to other typing methods (13, 50, 51). MLVA methods are in addition rapid and and not as expensive and laborious as SBT. Nevertheless, besides these advantages, MLVA usually lacks standardization that can complicate the comparison and interpretation of results. For instance, a single bacterial pathogen can be studied by different MLVA protocols generated by different laboratories or there may be differences in the sizing of amplicons due to different platforms or chemicals, in the nomenclature used to designate the genotypes or in the interpretation of incomplete repeats. Therefore, validation of the protocol, as recommended in recognized guidelines (26, 25) is crucial during the implementation of MLVA in a new laboratory. In the case of *L. pneumophila*, two MLVA protocols had been previously described (16, 18). The MLVA-8 protocol consisted

of a combination of eight VNTR markers formed by longer repeat sequences or minisatellites (Lpms1, Lpms3, Lpms13, Lpms19, Lpms33, Lpms34 and Lpms35). MLVA-12 protocol included 12 VNTR markers, of which seven were comprised in MLVA-8 and five new ones were added, one minisatellite (Lpms31) and four microsatellites (Lpms38, Lpms39, Lpms40 and Lpms44). Overall, general, the validation of the MLVA protocol in our laboratory confirmed the reproducibility and repeatability of the protocol, the specificity of the VNTR markers for *L. pneumophila* and the high discriminatory power.

The reproducibility of MLVA-8 and MLVA-12 schemes was tested by comparing the profiles of reference strains using capillary electrophoresis and sequencing of the PCR products for each marker. The sizes of the amplicons and therefore, the number of repeats for each marker, analyzed by sequencing corresponded with the expected sizes, or sizes observed *in silico* when the primers were designed. However, the sizes of the amplicons obtained by capillary electrophoresis differed in few basepairs from the expected sizes. This was observed in other studies (29, 30, 52, 53). The differences in basepairs increased with the number of repeats, specifically there was a difference of 21 bp less for Lpms31, which is the marker with highest number of repeats. The size differences were consistent and did not interfer when calculating the number of repeats, except for marker Lpms31. This could be due to large size of the amplicon that could have sequence-specific migration performance. Other reasons for differences between the observed and the theoretical sizes could be the size standard, type of polymer, or the instrument used. Since this only occurred with Lpms31 it appears to be intrinsic to this marker. It could be suggested that markers whose size differ from their expected size and can lead to inaccurate calculation of number of repeats could be excluded of the typing scheme, despite the high index of diversity.

A very good repeatability was determined for the MLVA-8 and MLVA-12 schemes after duplicate typing of 36 strains were carried out (**Table S4**). Usually, less than one basepair variation in fragment size was observed between different runs and therefore, the number of repeats was concordant for all markers. The typeability, or possibility of assign an isolate to a certain MLVA type, was as well very good since all isolates could be genotyped. Null alleles were observed for the VNTR markers Lpms1, Lpms19, Lpms38, Lpms40, at frequencies ranging from 0.67% in Lpms34 (microsatellite) to 18.12% in Lpms40 (minisatellite). However, no correlation has been found between null allele frequency and microsatellite repeat length nor motif complexity (54). Null alleles are considered a common genotyping error (55, 56). Although many studies using MLVA and microsatellites have reported the presence of null alleles (57–59). They are due to mutations in the annealing sequence, which produce poor amplicon or no amplification of the marker at all.

Regarding the index of discrimination of both MLVA typing schemes, MLVA-12 showed greater resolution than MLVA-8, as expected, due to the introduction of five highly

discriminative markers, especially Lpms31, Lpms38 and Lpms39, with 15, 8 and 12 alleles respectively. The combination of both schemes using in total 13 markers, so called MLVA-8(12), did not reveal a big improvement of the resolution compared to MLVA-12, since only two new genotypes were observed. Thus, typing separately Lpms17, the VNTR included in MLVA-8 but absent in MLVA-12, could be abandoned. The analysis of the 13 VNTRs regarding their index of diversity suggested a combination of nine markers that would maximize the discriminatory power and would resolve the same number of genotypes than using all 13 VNTRs. Nevertheless, the use of the 12 loci of the MLVA-12 scheme showed the highest congruence with SBT, a requirement for two typing methods that are used in a complementary manner.

An important fact to point out is the general lack of consensus about the type of rounding that should be selected for allele assignment. While some studies take into consideration half repeats to assign new alleles (18, 59), most of the MLVA studies preferred to make use of the rounding to the nearest whole integer, or to round down to consider exclusively complete repeats (13, 60–62). The second rounding strategy could be less biased and easier to reproduce than the rounding considering half sizes, unless it is well specified how to determine a half repeat. In this study half repeats have not been considered, in contrast to the previous MLVA protocols for *L. pneumophila* (18). However, to compare the newly obtained genotypes with the profiles downloaded from the database; those were as well rounded to the nearest integer and then compared. The instructions to follow for the allele assignment should be clearly specified when new MLVA protocols are designed to make them truly efficient and comparable.

In conclusion, it was demonstrated that the combined MLVA method described in this study was an appropriate typing method for *L. pneumophila* isolates due to the upright performance for the criteria discussed above. Nonetheless, its rapidity, costs efficiency, accessibility to technical resources (availability of a capillary sequencer) and the portability of the results make MLVA very suitable as genotyping method.

2.5.2 *L. pneumophila* diversity and population structure in Germany using MLVA analysis

In Germany, the population genetics of *L. pneumophila* isolates have been previously studied by using PFGE (63), SBT (1, 19) or by Variable genetic Element Typing (VET) (64). Nonetheless, no studies using MLVA genotyping for *L. pneumophila* have been reported in Germany. Thus, the study of the diversity of *L. pneumophila* isolates in Germany by MLVA could help to obtain insight into the population structure and consequently improve the surveillance and disease control for LD.

As shown before during the validation of the MLVA protocols, the discriminatory power of the combined scheme MLVA-8(12) was higher than the discrimination given by MLVA-8 when both protocols were applied to the population of *L. pneumophila* isolates in

Germany. By MLVA-8(12), and the same phenomenon was seen by MLVA-8, the majority of genotypes (70%) appeared as singlentones, while few genotypes, specifically four genotypes, among them Gt4(17) or Gt36(45), appeared very frequently. These genotypes correspond to ST1 and ST23, and represent globally distributed sequence types. The high diversity or number of singletones observed in *L. pneumophila* could be a consequence of the great capability that this bacterium has to exchange genetic material. The genome of *L. pneumophila* is highly dynamic, with recombination and horizontal gene transfer of mobile genetic elements as the two important factors in its evolution (33)**.** *L. pneumophila* are naturally competent bacteria (65, 66) and comparative genome analysis have shown that the genome of *L. pneumophila* can contain integrative plasmids, genomic islands and conjugation elements (67). It has been reported that *L. pneumophila* can interchange DNA not only within the species *L. pneumophila* (68) but also within the genus *Legionella* (69) and with their eukaryotic hosts (70). An example of exchange within the species is the gene cluster coding for the synthesis of the lipopolysaccaride (LPS) responsible of the serogroup 1, which has been detected in different *L. pneumophila* strains of different lineages, suggesting that the serogroup 1 LPS cluster can be transferred horizontally (69). Recombination is another mechanism that shapes the population structure and evolution of *L. pneumophila* (71, 72). However, besides the evidence for recombination and horizontal gene transfer, the population structure of *L. pneumophila* has been considered to be clonal due the high linkage disequilibrium showed by studies using typing methods (73, 74) and due to the presence of strains or clonal complexes with a global distribution. Visca et al. (2011) (31) showed signs of recombination in a study of the populations of *L. pneumophila* carried out by using MLVA genotyping. ST1, which corresponds to Gt4(17) and is represented by the strain Paris, is the most abundant strain in VACC1. It has been isolated very frequently from the environment as well as from patients of LD throughout France (75) and other European countries (76–78) including Germany (19) . It has been found in very different environments, such as hot springs, cooling towers or freshwater systems in Asian countries (79) and it is one of the most abundant strains found in Canada (37, 80). ST23 is usually reported together with ST1. The results obtained by MLVA in this study support the hypothesis of a clonal population of these previous studies. Not only few genotypes, as Gt4(17) (ST1), are distributed across the country but also the clonal complexes or VACCs appeared broadly distributed. In general, the VACCs did not show any correlation with sampling locations, with exception of the small VACC5-A, which was exclusively found in Warstein. The lack of correlation between the strains that comprise the clonal complexes and geographical characteristics was observed by Cazalet et al. (2008) (69). The overall results indicated that the population structure of *L. pneumophila* isolates from Germany results from the combined action of widespread clonal complexes and genotypes, i.e. Gt4(17) along with genetic differentiation at shorter geographic distances (VACC5-A).

To better implement satisfactory control measures that prevent and limit new infections, it is necessary to know the scope of variation among clinical and environmental *L. pneumophila* strains. In this respect, this study was concordant with earlier results. The percentage of sgp1 among clinical isolates was significantly higher than among environmental isolates and the majority of clinical isolates were subgrouped as MAb3/1 positive, in contrast to the environmental isolates, where MAb3/1 negative were more predominant. The same proportions were demonstrated for *L. pneumophila* populations in the UK (76, 81), France (82) and in the US (83). Until now, there is no complete explanation for the enhanced ability of sgp1 strains, and in particular MAb3/1 positive strains, to cause disease. Hypothetically, some strains of *L. pneumophila* could be especially virulent to humans, easily aerosolized, or more suited to colonization of freshwater distribution systems (84). In this study, the diversity observed in clinical and environmental isolates was very similar, in contrast to a previous analysis (81) that showed that diversity of the clinical isolates was significantly less than that of the environmental isolates. The same group published later how these differences, although still present, were smaller (76).

Another important point that was shown by using MLVA genotyping methods and agreed with what it was shown by SBT, was the little overlap between the two populations, clinical and environmental isolates. Only 13 out of 92 MLVA-8(12) genotypes were common among clinical and environmental isolates. The same effect was observed with the sequence types. Usually, common clinical STs are rarely found in the environment and viceversa (23, 76). Harrison et al. (2009) (76) suggested that well-managed freshwater systems present little risk to the human population, due to the low incidence of isolation of clinical strains from the environment. With the data set used in this study it is not possible to make the same suggestion. Among the common genotypes, Gt4(17), and therefore, ST1, was the most abundant in the two populations. Environmental and clinical Gt4(17) were related to community acquired and nosocomial cases of LD. Borchard et al. (2008) (19) described that ST1 was, by far, the most common ST in Germany among isolates from patients, although they noted that their sample of isolates was probably not representative of Germany as a whole, as nosocomial cases were very over-represented. The dataset used in this study might be biased due to the random selection of isolates but it pointed out that ST1 is also frequently present in different environments in Germany. Not only in Germany but in many other countries ST1 appeared as the most prevalent ST for both clinical sporadic and environmental *L. pneumophila* sgp1 group isolates.

Until today, there is no confirmed explanation to the presence of globally distributed clones. A hypothesis could be that these clones are easier cultured than other strains, indicating bias in the isolation procedures. However, Sánchez-Busó et al. (2014) (72), showed a high congruence in the level of diversity detected by molecular methods and by SBT based on isolates. Geographical barriers do not appear to be significant for the formation of the clones. It

could be more suitable that the strains or group of strains that form the extensively distributed clonal complexes would be adapted to specific ecological niches. Amemura-Maekawa et al. (2005) (85) and Cohan et. Al (2007) (86) reported the presence of possible stable ecotypes adapted to specific ecological environments. More recently, in a study where environmental strains of *L. peumophila* were genotyped by MLVA-8 by Rodríguez-Martínez et al. (2015) (87), it was suggested that distinct genotypes (Gt4 and Gt6) included within clonal complex VACC1 could be perceived as different ecotypes with features rendering them competent in different niches. Furthermore, whole genome analyses and physiological studies open new research avenues to reveal if specific genes could contribute to improved fitness in the specific environment.

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2.7 Supplementary material

Table S1. Characteristics of the VNTR loci and primer sequences used in this study.

VNTR	Primer sequence (5´-3´)	Repeat unit size (bp)	Position (bp)[1]	MLVA scheme	Associated gene	Function	Reference
Lpms01	GCATATGACAAAGCCTTGGC	45	3231393	MLVA-8/	lpg2854	Hypothetical protein	Sobral et al. 2011
	NED-TGAATTTCTCCCTCTTGCTTG			MLVA-12			
Lpms03	TGATGGTCTCAATGGTTCCG	96	3145527	MLVA-8/	lpg2793	Protein LepA, onserved gene	Sobral et al. 2011
	VIC-GGACAAACAACCAATGAAGC			MLVA-12			
Lpms13	GCATCGGACTGAGCAAAGTA	24	1645025	MLVA-8/	lpg1488	Conserved protein, surface antigen	Sobral et al. 2011
	NED-CTCACCAGGATGCTTTGTCG			MLVA-12			
Lpms17	TAACATCAATGACCGCGAAA	39	931165	MLVA-8	lpg0854	Hypothetical protein	Pourcel et al. 2007
	VIC-CAGCTCACCCCGTATCACTT						
Lpms19	TCCAGAGGCTCTGGATTATC	21	913445	MLVA-8/	Intergenic		Sobral et al. 2011
	VIC-GAACTATCAGAAGGAGGCGA			MLVA-12			
Lpms31	ATCGCCTAATTGCCGCCTA	45	2991504	MLVA-12			Sobral et al. 2011
	FAM-CCTCGCAAGCCTATGTGG						
Lpms33	CGAGGAAATCTTCTTCAGCC	125	2578937	MLVA-8/	Intergenic		Sobral et al. 2011
	VIC-GACACCACAGCAGTTTGAAC			MLVA-12			
Lpms34	ATGCAGGATGTTTGCGCATG	125	2662138	MLVA-8/	lpg2356	Transmembrane protein	Sobral et al. 2011
	FAM-AAGGAATAAGGCGCAGCAC			MLVA-12			
Lpms35	TATCAACCTCATCATCCCTG	18	1428473	MLVA-8/	lpg1299	Protein FimV,	Sobral et al. 2011
	PET-GAATCTGAAACAGTTGAGGATG			MLVA-12		transmembrane Tfp pilus assembly	
Lpms38	GGATTGCCTTGGGCATTAAT	8	857393	MLVA-12	lpg0692	ABC type transport, ATPase component	Sobral et al. 2011
	NED-CCTATCAACAGATGACGCTT						
Lpms39	CCAACTCCTCAACGCAACAA	6	3219356	MLVA-12	lpg2844	Hypothetical histidine-rich protein	Sobral et al. 2011
	PET-CTTGACGAAGTAGGTGTGGG						
Lpms40	TTACCCAAGCCCTTATTGCG	6	29936	MLVA-12	lpg0023	Transmembrane protein	Sobral et al. 2011
	FAM-TAGATCTCTTGCCGAGCTTC						
Lpms44	TTATGCGAGAGTTTCATGA	6	3009948	MLVA-12	lpg0321	50S ribosomal protein	Sobral et al. 2011
	NED-GCTACTGCAGCAACATCC						

[1] Position on the *L.pneumophila* Philadelphia-1 genome

Table S2. *Legionella* strains isolated at the HZI campus between June 2013 and July 2014.

Strain Designation	Sgp	*Leg.* genus 16S rRNA PCR	*L.pn.* 16S rRNA PCR	Site of isolation	Type of sample	Isolation date	Closest described bacterial species (% similarity, accession no.)
H16	2-14	+	+	Cooling tower	Bulk water	19/06/2013	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H17	2-14	+	+	Cooling tower	Bulk water	19/06/2013	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H18	1	+	+	Cooling tower	Biofilm	19/06/2013	100% Legionella pneumophila str. Paris complete genome CR628336.1
H19	2-14	+	+	HZI (Kitchen faucet D2)	Biofilm	28/06/2013	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H20	2-14	+	+	HZI (Kitchen faucet D2)	Biofilm	28/06/2013	99% Legionella pneumophila subsp. pneumophila str. Lorraine chromosome, FQ958210.1
H21	1	+	+	HZI (Kitchen faucet D2)	Biofilm	28/06/2013	99% Legionella pneumophila str. Paris complete genome CR628336.1
H22	2-14	+	+	HZI (Kitchen faucet D2)	Biofilm	28/06/2013	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H23	2-14	+	+	HZI (Kitchen faucet D2)	Biofilm	28/06/2013	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H24	2-14	+	+	Cooling tower	Biofilm	01/11/2013	100% Legionella pneumophila subsp. pneumophila str. Lorraine chromosome, FQ958210.1
H25	1	+	+	Cooling tower	Biofilm	01/11/2013	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H26	2-14	+	+	Cooling tower	Biofilm	01/11/2013	100% Legionella pneumophila subsp. pneumophila str. Lorraine chromosome, FQ958210.1
H27	1	+	+	Cooling tower	Biofilm	01/11/2013	100% Legionella pneumophila subsp. pneumophila str. Lorraine chromosome, FQ958210.1
H28	2-14	+	+	Cooling tower	Bulk water	28/11/2013	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H29	2-14	+	+	Cooling tower	Bulk water	28/11/2013	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H30	2-14	+	+	Cooling tower	Bulk water	28/11/2013	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H31	2-14	+	+	Cooling tower	Bulk water	28/11/2013	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H32	2-14	+	+	Cooling tower	Bulk water	28/11/2013	99% Legionella pneumophila str. Corby, complete genome CP000675.2
H33	2-14	+	+	Cooling tower	Bulk water	28/11/2013	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H34	2-14	+	+	Cooling tower	Bulk water	28/11/2013	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H35	1	+	+	Cooling tower	Bulk water	28/11/2013	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H36	2-14	+	+	Cooling tower	Bulk water	28/11/2013	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H37	2-14	+	+	Cooling tower	Bulk water	28/11/2013	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H38	1	+	+	Cooling tower	Bulk water	28/03/2014	100% Legionella pneumophila str. Paris complete genome CR628336.1
H39	2-14	+	+	Cooling tower	Bulk water	28/03/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H40	2-14	+	+	Cooling tower	Bulk water	28/03/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1

H41	2-14	+	+	Cooling tower	Bulk water	28/03/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H42	1	+	+	Cooling tower	Bulk water	28/03/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H43	2-14	+	+	Cooling tower	Bulk water	28/03/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H44	2-14	+	+	Cooling tower	Bulk water	28/03/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H45	2-14	+	+	Cooling tower	Bulk water	28/03/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H46	2-14	+	+	Cooling tower	Bulk water	28/03/2014	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H47	2-14	+	+	Cooling tower	Bulk water	28/03/2014	100% Legionella pneumophila str. Corby, complete genome CP000675.2
H48	1	+	+	Cooling tower	Bulk water	28/03/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H49	negative	+	-	HZI (Men´s toilet D0.21)	Hot water	08/04/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H50	2-14	+	+	HZI (Men´s toilet D0.21)	Hot water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H51	2-14	+	+	HZI (Men´s toilet D0.21)	Hot water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H52	1	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H53	1	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H54	negative	+	-	Cooling tower	Bulk water	08/04/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H55	negative	+	-	Cooling tower	Bulk water	08/04/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H56	negative	+	-	Cooling tower	Bulk water	08/04/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene,partial sequence AY444741.1
H57	2-14	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H58	2-14	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H59	2-14	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H60	2-14	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H61	2-14	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H62	1	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H63	2-14	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H64	2-14	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H65	negative	+	-	Cooling tower	Bulk water	08/04/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H66	negative	+	-	Cooling tower	Bulk water	08/04/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H67	negative	+	-	Cooling tower	Bulk water	08/04/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H68	negative	+	-	Cooling tower	Bulk water	08/04/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H69	2-14	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H70	2-14	+	+	Cooling tower	Bulk water	08/04/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H71	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1

H72	1	+	+	Cooling tower	Bulk water	25/06/2014	100% Legionella pneumophila str. Lens complete genome CR628337.1
H73	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H74	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H75	2-14	+	+	Cooling tower	Bulk water	25/06/2014	99% Legionella pneumophila subsp. fraseri strain Los Angeles-1 16S ribosomal RNA gene NR_104921.1
H76	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H77	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H78	2-14	+	+	Cooling tower	Bulk water	25/06/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H79	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H80	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H81	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H82	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H83	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H84	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H85	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H86	negative	+	-	Cooling tower	Bulk water	25/06/2014	99% Legionella longbeachae strain ATCC 33484 16S ribosomal RNA gene, partial sequence AY444741.1
H90	2-14	+	+	Cooling tower	Bulk water	13/07/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H91	2-14	+	+	Cooling tower	Bulk water	13/07/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1
H92	2-14	+	+	Cooling tower	Bulk water	13/07/2014	100% Legionella pneumophila subsp. pneumophila str. Philadelphia 1 AE017354.1

Table S3. Reproducibility test by comparison PCR product length inferred by capillary electrophoresis and sizes from the *L. pneumophila* allele assignment table previously published. MLVA-8 markers (plus Lpms31) were compared for *L. pn*. Paris and *L. pn. Lens*. MLVA-12 markers were compared for *L. pn*. Philadelphia-1. VNTR markers of *L. pn.* Philadelphia-1 were as well sequenced and compared.

	***L.pn*. Philadelphia-1**			***L.pn*. Paris**		***L.pn*. Lens**	
VNTR	**Expected size (bp) (no. of repeats)[1,3]**	**Sequencing (bp) (no. of repeats)[2]**	**CE (bp) (no. of repeats)[2]**	**Expected size (bp) (no. of repeats)[3]**	**CE (bp) (no. of repeats)[2]**	**Expected size (bp) (no. of repeats)[3]**	**CE (bp) (no. of repeats)[2]**
Lpms01	498 (8)	494 (8)	503 (8)	520 (7)	516 (7)	475 (6)	476 (6)
Lpms03	942 (8)	941 (8)	930 (8)	845 (7)	843 (7)	845 (7)	840 (7)
Lpms13	793 (11)	793 (11)	784 (11)	404 (10)	400 (10)	260 (4)	262 (4)
Lpms17	278 (2)	275 (2)	274 (2)	278 (2)	275 (2)	278 (2)	276 (2)
Lpms19	128 (4)	128 (4)	128 (4)	173 (4)	171 (4)	173 (4)	171 (4)
Lpms31	1043 (17)	1035 (17)	1017 (16)	615 (9.5)	610 (10)*	795 (13.5)	797 (14)*
Lpms33	317 (1)	314 (1)	313 (1)	604 (4)	603 (4)	352 (2)	350 (2)
Lpms34	265 (1)	266 (1)	259 (1)	334 (2)	337 (2)	460 (3)	460 (3)
Lpms35	205 (3)	204 (3)	203 (3)	454 (17)	459 (17)	562 (23)	558 (23)
Lpms38	264 (3)	258 (3)	260 (3)				
Lpms39	79 (6)	77 (6)	80 (6)				
Lpms40	198 (4)	196 (4)	196 (4)				
Lpms44	173 (9)	174 (9)	169 (8)				

[1] As published by Sobral et al. 2011 sand Pourcel et al. 2007

[2] Result obtained in this study

[3] As published by Pourcel et al. 2007

* The number of repeats are rounded to the nearest integer value in this study

Table S4. Repeatability test of the MLVA-12 multiplex PCR and Lpms17 singleplex PCR and allele size measurement (bp) by capillary electrophoresis using 36 strains included in panel I.

Strain designation	Test	Lpms1	Lpms3	Lpms13	Lpms17	Lpms19	Lpms31	Lpms33	Lpms34	Lpms35	Lpms38	Lpms39	Lpms40	Lpms44
L. pn. Philadelphia-1	PCR1	503.55	930.14	784.00	274.09	122.80	1017.80	313.29	259.10	202.98	260.33	80.55	196.00	169.21
ATCC 33152T	PCR2	503.39	930.35	784.22	274.68	122.97	1018.08	313.37	259.18	202.99	259.09	80.60	196.32	169.24
L. pn. Bloomington-2	PCR1	504.20	930.99	760.54	274.03	144.80	0.00	684.51	260.62	344.80	504.20	104.80	196.53	169.39
ATCC 33155	PCR2	503.58	930.17	761.19	273.90	144.02	0.00	684.45	260.37	344.18	503.87	105.09	196.15	169.23
L. pn. Paris	PCR1	457.48	835.60	761.60	275.54	122.90	693.10	687.40	383.70	450.60	260.40	128.40	202.60	151.30
CIP 107629	PCR2	457.50	835.48	761.26	275.98	123.92	692.34	687.23	383.51	450.93	260.21	128.22	202.45	151.10
L. pn Chicago-2	PCR1	503.50	929.90	784.10	273.73	143.60	1018.00	313.50	259.20	203.10	260.00	80.60	196.50	169.20
ATCC 33215	PCR2	503.39	930.06	784.22	274.49	144.26	1018.08	313.37	259.18	202.99	259.09	80.60	196.32	169.24
L. pn. Corby	PCR1	0.00	930.00	737.00	274.98	124.00	817.30	685.50	260.20	573.00	260.00	127.70	196.20	169.00
	PCR2	0.00	929.62	736.35	274.65	123.67	816.81	684.98	260.87	572.97	260.67	128.15	196.49	169.45
L13-435	PCR1	503.80	930.27	713.17	274.00	143.64	879.80	437.37	384.54	256.40	260.17	105.13	196.24	169.27
	PCR2	504.02	930.02	713.45	275.68	143.61	879.49	437.42	384.58	256.39	260.24	105.05	196.17	169.33
L13-444	PCR1	503.85	930.12	713.53	273.43	143.64	879.61	437.40	384.59	256.43	260.21	105.13	196.27	169.27
	PCR2	504.34	930.67	713.47	274.19	143.33	879.85	437.34	384.02	256.76	260.29	105.85	196.86	169.03
L13-445	PCR1	503.82	930.09	713.50	274.68	143.61	879.58	437.37	384.56	256.40	260.18	105.10	196.24	169.24
	PCR2	504.28	930.61	713.41	274.35	143.27	879.79	437.28	383.96	256.70	260.23	105.79	196.80	168.97
L13-446	PCR1	504.47	930.47	713.69	273.70	143.78	879.83	437.11	384.09	256.24	260.16	105.47	196.01	169.14
	PCR2	504.56	930.56	713.78	275.38	143.87	879.92	437.20	384.18	256.33	260.25	105.56	196.10	169.23
W13-845-1	PCR1	457.00	929.83	713.45	274.29	144.72	816.93	437.32	384.58	344.53	260.21	0.00	196.26	169.28
	PCR2	457.49	929.26	713.79	274.78	144.81	817.06	437.32	384.63	344.64	260.32	0.00	196.60	169.35
W13-845-2	PCR1	503.32	929.82	736.45	274.45	123.53	879.60	437.08	384.36	520.56	259.79	104.80	195.75	168.87
	PCR2	503.77	930.27	736.90	275.02	123.98	880.05	437.53	384.81	521.01	260.24	105.05	196.20	169.32
W13-845-3	PCR1	456.97	929.80	713.42	274.30	144.69	816.90	437.29	384.55	344.50	260.18	0.00	196.23	169.29
	PCR2	457.45	929.29	712.98	274.18	144.45	817.63	436.82	384.08	343.13	260.39	0.00	195.67	169.03

W13-845-4	PCR1	503.62	930.10	713.36	274.06	143.46	879.04	437.23	384.29	256.36	260.12	105.10	195.94	169.11
	PCR2	503.95	929.95	713.38	273.94	143.54	879.42	437.35	384.51	256.32	260.17	104.98	196.10	169.26
W13-845-5	PCR1	456.94	929.77	713.39	274.52	144.66	816.87	437.26	384.52	344.47	260.15	0.00	196.20	169.22
	PCR2	457.49	929.26	713.79	273.87	144.81	817.06	437.32	384.63	344.64	260.32	0.00	196.60	169.35
W13-845-6	PCR1	503.88	930.20	736.94	274.37	122.85	879.72	437.41	383.96	520.87	260.25	105.09	196.22	169.27
	PCR2	503.80	930.12	736.86	273.72	122.55	879.12	437.34	383.34	521.90	260.23	104.78	198.39	170.69
W13-845-7	PCR1	504.67	931.24	737.35	273.93	124.65	881.13	438.15	385.00	522.47	261.80	106.29	197.84	170.62
	PCR2	503.77	930.34	736.45	274.14	123.75	880.23	437.25	384.10	521.57	260.90	105.39	196.94	169.72
W13-845-8	PCR1	457.50	930.06	713.44	274.35	144.76	817.30	437.34	384.57	344.59	260.20	0.00	196.20	169.27
	PCR2	457.49	929.26	713.79	274.56	144.81	817.06	437.32	384.63	344.64	260.32	0.00	196.60	169.35
L13-438	PCR1	504.01	929.97	713.49	274.77	143.49	879.78	437.34	384.58	256.43	260.21	105.05	196.23	169.23
	PCR2	504.02	930.02	713.45	274.98	143.61	879.49	437.42	384.58	256.39	260.24	105.05	196.17	169.33
L13-443	PCR1	503.89	930.15	783.96	275.19	0.00	1017.80	313.29	259.10	202.99	0.00	80.56	196.51	169.21
	PCR2	503.72	930.20	784.30	274.81	0.00	1018.32	313.50	259.33	203.06	0.00	80.50	196.42	169.35
W13-845-11	PCR1	457.35	929.77	713.22	274.65	143.50	816.02	437.08	384.34	344.46	260.00	129.36	196.20	169.10
	PCR2	457.56	929.34	713.87	274.49	143.34	816.86	437.20	384.39	344.29	260.89	128.98	196.83	169.89
W13-845-12	PCR1	457.45	930.94	713.34	274.33	144.09	817.34	437.72	384.98	346.00	260.22	0.00	196.03	169.00
	PCR2	457.52	929.29	713.82	274.44	144.84	817.09	437.35	384.66	344.67	260.35	0.00	196.63	169.38
W13-845-13	PCR1	503.80	930.13	736.75	274.55	122.80	879.88	437.47	383.91	520.95	260.21	105.05	196.22	169.23
	PCR2	503.12	930.83	736.01	274.57	122.63	878.92	437.29	383.14	520.18	260.10	105.91	196.11	169.61
W13-845-14	PCR1	457.74	930.27	713.91	274.68	144.30	817.23	437.29	384.21	344.12	260.83	0.00	196.92	169.09
	PCR2	457.48	929.26	713.79	274.70	143.26	816.78	437.12	384.31	344.21	260.81	0.00	196.75	169.81
W13-845-15	PCR1	457.34	930.90	713.14	274.81	144.89	817.71	437.94	384.82	344.29	260.23	0.00	196.92	169.63
	PCR2	457.49	929.26	713.79	274.83	144.81	817.06	437.32	384.63	344.64	260.32	0.00	196.60	169.35
W13-845-16	PCR1	565.77	930.05	713.68	274.43	122.83	817.26	561.55	505.67	468.07	260.42	105.11	196.19	169.25
	PCR2	565.02	930.34	713.82	274.54	122.02	817.29	561.34	505.82	468.29	260.92	105.92	196.26	169.82
W13-845-18	PCR1	457.50	930.06	713.44	274.56	144.76	817.30	437.34	384.57	344.59	260.20	0.00	196.20	169.27

	PCR2	457.64	929.20	713.79	274.67	144.40	817.71	437.97	384.32	344.78	260.02	0.00	196.64	169.35
W13-845-19	PCR1	457.29	930.09	713.31	274.69	143.50	816.10	437.18	384.31	344.40	260.04	131.18	195.95	169.13
	PCR2	457.34	929.35	713.30	274.67	144.67	817.24	437.20	384.90	344.30	260.52	131.83	196.92	169.02
W13-845-20	PCR1	457.45	930.91	713.69	274.59	144.19	817.71	437.34	384.45	344.20	260.90	0.00	196.34	169.01
	PCR2	457.96	929.26	713.51	274.51	144.90	817.16	437.97	384.01	344.00	260.91	0.00	196.82	169.97
W13-845-21	PCR1	457.50	930.98	713.44	274.70	144.76	817.30	437.34	384.57	344.59	260.20	0.00	196.20	169.27
	PCR2	457.24	929.33	713.34	274.81	144.56	817.20	437.23	384.04	344.82	260.82	0.00	196.82	169.02
W13-845-22	PCR1	457.50	930.06	713.44	274.93	144.76	817.30	437.34	384.57	344.59	260.20	0.00	196.20	169.27
	PCR2	457.49	929.26	713.79	274.87	144.81	817.06	437.32	384.63	344.64	260.32	0.00	196.60	169.35
W13-845-23	PCR1	457.89	930.24	713.87	274.79	144.78	817.28	437.89	384.12	344.09	260.46	0.00	196.64	169.08
	PCR2	457.46	929.92	713.92	274.71	144.34	817.34	437.84	384.92	344.92	260.00	0.00	196.01	169.89
W13-845-25	PCR1	457.37	930.06	713.55	274.90	144.64	815.99	437.14	384.23	344.42	260.08	0.00	195.96	169.09
	PCR2	457.16	929.25	713.26	275.01	144.48	817.12	437.15	383.96	344.74	260.74	0.00	196.74	168.94
W13-845-26	PCR1	457.75	930.75	713.23	275.13	144.08	817.91	437.08	384.91	344.73	260.23	0.00	196.00	169.97
	PCR2	457.33	930.13	713.35	274.94	143.54	816.14	437.22	384.35	344.44	260.08	0.00	195.99	169.17
W13-845-27	PCR1	457.50	930.06	713.44	274.86	144.76	817.30	437.34	384.57	344.59	260.20	0.00	196.20	169.27
	PCR2	457.43	929.99	713.37	275.05	144.69	817.23	437.27	384.50	344.52	260.13	0.00	196.13	169.20
W13-845-28	PCR1	457.74	929.43	713.67	275.16	143.28	815.92	437.02	304.23	344.09	260.00	131.21	195.92	169.11
	PCR2	457.49	929.26	713.79	275.28	144.81	817.06	437.32	384.63	344.64	260.32	131.83	196.60	169.35
W13-845-31	PCR1	457.34	930.34	713.67	274.00	144.67	817.24	437.94	384.93	344.02	260.62	0.00	196.00	170.00
	PCR2	457.77	929.46	713.70	274.21	143.31	815.95	437.05	304.26	344.12	260.03	0.00	195.95	169.14

Chapter III

Characterization of populations of *L. pneumophila* isolates in the Middle East and comparison with populations in Central Europe

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3.1 Abstract

Legionella pneumophila is a water-based bacterium responsible for most cases of legionellosis, a atypical pneumonia with an increasing incidence in industrialized countries. The study of the genetic diversity and the geographical distribution of *L. pneumophila* strains are essential for the development of public health control strategies, especially in areas where not much epidemiological information is available. Genotyping methods, such as Sequence Based Typing (SBT) and Multi Locus Variable number of tandem repeats Analysis (MLVA), are current approaches used in the identification of *L. pneumophila* strains. In this study, MLVA was selected, due to its high resolution and rapidity, to characterize a total of 431 *L. pneumophila* strains isolated from water and biofilm samples across the West Bank and Northern Israel. Most of the environmental isolates in the two areas were identified as serogroup 1 (83.3% and 62.3% of the isolates in Israel and West Bank, respectively). Remarkably, besides the geographical proximity, only two genotypes were common to Israel and West Bank, which corresponded to the most prevalent genotypes, Gt4(17) and Gt6(18). Particularly, Gt4(17) and Gt6(18) were the most frequently isolated genotypes from the environment (they represented together the 49.9% and 57.8% of environmental isolates in Israel and West Bank, respectively) as well as from patients affected of pneumonia in Israel (54.5%). These genotypes corresponded to Sequence Type 1 (ST1), a common worldwide distributed clonal complex. Only three genotypes of *L. pneumophila,* of which two corresponded to ST1, were shared among the Middle East and Europe. This study showed the biogeography of *L. pneumophila* strains in the Middle East and remarkable differences to European strains.

3.2 Introduction

Besides the significance of the *L. pneumophila* as a water-based pathogen responsible of producing a severe, and often fatal, pneumonia in people whose immune defenses are weakened, only few studies have been carried out to our knowledge to evaluate the impact of this bacterium in the Middle East. Studies carried out in Europe and USA have revealed an increase in the incidence of Legionnaires' disease (LD) in the last years (1–3). Studies performed in the Middle East have reported the prevalence of *L. pneumophila* in freshwater systems and assessed their epidemiology by using mostly culture-dependent methods (4, 5). More recently, a study reported the presence of *L. pneumophila* in air conditioned buildings in Kuwait by using molecular techniques (6).

Only a limited amount of data are available about the epidemiology of *L. pneumophila* in the area of Israel (7–9). It was only two years ago when a study described the molecular epidemiology of *L. pneumophila* by Sequence Based Typing (SBT) in Israel (10) and Rodríguez-Martínez et al. (2015) (11) described more recently the clonal population of a set of environmental isolates of *L. pneumophila* obtained in a drinking water distribution system at the Oranim campus in the city of Kyriat Tivon in Northern Israel. In the area of West Bank, no studies regarding the presence and the populations of *L. pneumophila* have appeared yet.

The study by Moran-Gilad et al. (2014) (10) showed that the epidemiological tendencies of LD in Israel were similar to the tendencies previously shown in the EU. The majority of clinical isolates were identified as *L. pneumophila* serogroup 1 (71.4%). However, isolates that belonged to serogroup 3 were as well responsible of causing infections (14.2%) and this serogroup was also present among the environmental isolates from drinking water samples in Israel (9). According to the molecular assessment of the epidemiology, ST1, the most worldwide dispersed sequence type, was the most common among clinical and environmental strains in Israel (10). The common presence of serogroup 3 strains among environmental as well as clinical isolates in Israel, in contrast to observations in EU (12, 13), the identification of new strains and the high dominance of ST1 suggested additional analysis. Especially increasing the sample size and the applying high resolution molecular typing methods could help to further characterize ST1.

Multi Locus Variable number of tandem repeats Analysis (MLVA) genotyping has exhibited high resolution in comparison to other typing methods for a variety of bacterial species (14–16). In the case of *L. pneumophila,* MLVA genotyping presented higher discrimination than SBT (17, 18) and a very high concordance with this current gold standard method as described by Pourcel et al. (2007) and Visca et al. (2011) (17, 18) as well as presented previously in Chapter II of this thesis. These criteria together with the rapidity, the high typeability and reproducibility as well as the existence of a large database made MLVA genotyping the method of choice for the study of the populations of *L. pneumophila* isolates in

the Middle East. The study of the genetic diversity and distribution of *L. pneumophila* strains, especially in areas were not much information is available could be essential for later epidemiological studies and for public health control strategies.

The aim of this study was to assess the genetic diversity and the distribution of *L. pneumophila* strains in the Middle East by MLVA high resolution genotyping using a large dataset composed of 431 environmental and clinical isolates obtained during the period of 2012-2014 in Northern Israel and along the West Bank. The genetic diversity of the two areas in the Middle East was compared and a further evaluation was carried out by comparing with the diversity observed in central Europe using strains from Germany, previously described in Chapter II, and the International MLVA *Legionella* database.

3.3 Material and methods

3.3.1 Water sampling and *Legionella* isolation in Israel

Water sampling was carried out by our collaborating partners from the University of Haifa around the campus of Oranim, at Kiryat Tivon, Northern Israel, since summer 2012 until autumn 2013 (**Table S1, Figure S1**). Lake Kinneret water as well as ground water is provided as drinking water in Israel. Briefly, seven points were selected covering the water rout at the campus. Biofilm and cold water samples were taken seasonally at all sampling points. Hot water samples were taken when available. *Legionella* isolation from biofilm and water samples was performed based on ISO 11731:1998 (International Organization for Standardization, 1998) and as described by Rodríguez-Martínez et al. (2015) (11). Additionally to the bacterial isolation, biotic and abiotic analysis of the water, as heterotrophic plate counts or measurement of temperature, pH or chlorine, were carried out for each sample (11). Furthermore, the same *Legionella* isolation procedure was performed for samples taken at eight different locations across Northern Israel during spring and summer 2013. They were mostly private homes situated in the localities of Kyriat Tivon (Raz and Tamar), Alonei Abba, Yavne´el, Mehanamia, Tiberias (Hila and Shosh) and Moshav (Arbel). In addition, one of the sampling sites was located at the campus of the Technion University in Haifa (**Table S1**, **Figure S2**). Besides the environmental isolates obtained during the sampling, 11 *L. pneumophila* clinical isolates obtained from patients affected of pneumonia were kindly provided by the Rambam hospital in Haifa.

3.3.2 Water sampling and *Legionella* isolation along the West Bank

A sampling campaign was performed by our partners from Al-Quds University in Jerusalem along the West Bank during two years (2012-2014). Water and biofilm samples were taken from eight hospitals along the West Bank: hospitals A, B and C located in Northern West

Bank; hospitals D, E and F in the central area of West Bank and hospitals G and H located in Southern West Bank (**Table S1**, **Figure S2**). Moreover, samples were as well taken from Al-Quds University main campus located in Jerusalem, central West Bank. At the hospitals, water and biofilm samples were taken from kitchens and toilets but also in showers and faucets situated in areas occupied by high risk patients, as the Intensive Care Unit, surgery and emergency rooms, as well as areas of oncology. *Legionella* isolation from water and biofilm samples was based on ISO 11731:2004 (19). Biotic and abiotic analyses of the water were additionally carried out as described previously (11).

3.3.3 Phenotypic and molecular characterization of *L. pneumophila* isolates

Species identification of the *L. pneumophila* isolates obtained in Israel and West Bank was carried out by serotyping and by 16S rRNA *Legionella*-genus and *L. pneumophila*-species specific PCR. Both PCRs were carried out by using primers and conditions described in Material and Methods of Chapter II. Serotyping was performed using a latex agglutination test. For isolates from the West Bank, the same procedure was followed as described in Chapter II for the isolates obtained in Germany. Strains isolated in Israel were serotyped using a different protocol that allowed to distinguish serogroup 3, besides serogroup 1 and serogroups 2-14 as described previously (11).

L. pneumophila isolates were sent to the Helmholtz Centre for Infection Research (HZI) in Braunschweig for further molecular characterization. A total of 251 *L. pneumophila* environmental and clinical isolates obtained in Israel were sent on FTA cards (Whatman, Germany) or just as pure extracted DNA dissolved in buffer. From the West Bank, a set composed of 180 *L. pneumophila* environmental isolates were sent as fixed on FTA cards and a small subset as living biomass streaked out in BCYE (Buffered Charcoal Yeast Extract Agar, Oxoid Thermoscientific, Germany) plates (**Table S1**).

For DNA extraction out of the FTA cards, the area of the card containing the biomass was punched with a puncher into 3 mm circular punches. Punches were transferred to 0.5 ml steril water (Roth, Germany). They were incubated for 3 min at room temperature and vortexed three times (after water addition, after 1 min and after 3 min incubation). The FTA punch was removed and 1x Tris-EDTA buffer (Sigma-Aldrich, Germany) was added to the water to preserve the DNA from degradation. DNA was finally quantified by using Picogreen (Molecular Probes, Invitrogen) and kept frozen at –20°C for later analyses.

MLVA-8 and MLVA-12 molecular genotyping assays by capillary electrophoresis were carried out for all isolates as detailed in Material and Methods of Chapter II. Representative isolates from West Bank and Israel were sent to the National Reference laboratory for *Legionella* infections in Dresden and were additionally analyzed by sequence based typing and by monoclonal antibody subgrouping (12, 20).

3.3.4 Statistical analysis

Capillary electrophoresis data analysis and calculation of the number of repeats for each VNTR marker were achieved as described in Chapter II. The numerical code used to designate MLVA-8 and MLVA-12 genotypes, as well as the joint code for MLVA-8(12) genotypes, was continued for the Middle East isolates. Null alleles ("0") were assigned when no amplicon was detected. Clustering analysis was performed in Bionumerics (version 5.0, Applied Maths). UPGMA (Unweighted Pair Group Method with Arithmetic Mean) method applying a categorical coefficient was used to define the clusters. MLVA-8 profiles obtained in this study were compared to those from the international *Legionella* database ((http://bacterial-genotyping.igmors.u-psud.fr/ Legionella2006) and clusters were defined applying as criteria a cut-off of 60% similarity as previously done (21). Minimum spanning trees were performed using the categorical coefficient. Simpson's Index of Diversity coefficient was calculated using the online tool provided in http://darwin.phyloviz.net/ComparingPartitions/. To measure the variation of the number of repeats at each VNTR locus, the Hunter-Gaston Discrimination Index (HGDI), which is a modification of the Simpson's Index of Diversity, was calculated using http://www.hpa-bioinfotools.org.uk/cgi-bin/DICI/DICI.pl. The online tool http://www.cmbi.ru.nl/cdd/biovenn (22) was used to create Venn diagrams for the comparison of strains among the distinct geographical areas under study. Google Earth (version 7.1.5.1557) was used to generate a visual representation of the selected sampling locations.

3.4 Results

3.4.1 Genetic diversity of *L. pneumophila* strains from the West Bank

A total of 180 *L. pneumophila* environmental isolates from seven hospitals and the campus of the Al-Quds University from all across West Bank were received at the HZI (**Table S1**). 175 isolates were obtained from biofilm samples, while only five isolates could be isolated directly from water. The majority of the isolates were characterized as serogroup 1 (sgp1) (n=112, 62.3%) (**Table 1**). A subset of 10 sgp1 isolates was subgrouped by monoclonal antibody and all belonged to the MAb 3/1 negative OLDA subtype. A third part of the isolates (n=68, 37.7%) were serotyped as non-sgp1. The specific serogroup of the 54.4% (n=37) of the non-sgp1 isolates was additionally analyzed by monoclonal subgrouping and 81% (n=30) of them were serotyped as sgp6, followed by sgp8 (n=6) and sgp10 (n=1). The rest of the non-sgp1 was just characterized as serogroups 2-14 as performed by the agglutination kit.

MLVA-8, as well as MLVA-12 and the joint scheme of MLVA-8(12) genotyping, were carried out to study of the population of *L. pneumophila* in the West Bank (**Table 2**). The 180 isolates were typed in 16 MLVA-8 genotypes (ID=0.771, 95% CI 0.721-0.822), 25 MLVA-12 genotypes (0.790, 95% CI, 0.739-0.841) and 26 MLVA-8(12) genotypes (ID=0.790, 95% CI 0.739-0.841). 92.8% (n=167) of the MLVA-8(12) genotypes were found at least twice while, only 7.2% (n=13) were found once. About 58% of the strains belonged to only two genotypes. In particular, the most frequent isolated genotype was Gt4(17) (n=71, 41.1%), followed by Gt6(18) (n=30, 16.7%). Both of them corresponded to ST1. The following most frequent genotypes were Gt10(93) (n=16, 8.9%), and Gt9(92) (n=8, 4.4%), corresponding to ST461, and Gt13(72) (n=10, 5.6%), which corresponded to ST1326.

Table 1 Serogroup and monoclonal antibody subtyping of 180 environmental *L. pneumophila* isolates from West Bank.

Serogroup	**MAb subgroup**	**Environmental isolates**	
		No.	**Frequency (%)**
1	OLDA	10	5.6
1	NA[1]	102	56.7
Total sgp1		*112*	*62.3*
6	Dresden	30	16.7
8		6	3.3
10		1	0.6
(2-14)		31	17.2
Total non sgp1		*68*	*37.7*
Total		*180*	*100*

[1]NA: Not analyzed

When applying MLVA-8(12) genotyping 96.6% of isolates (n=174) were clustered into four MLVA clonal complexes or VACCs (VACC1, VACC2, VACC5 and VACC11) (**Figure S3, Figure 1**) and the rest 3.6% (n=6) were found as individual genotypes. VACC1, VACC2 and VACC5 were clonal complexes previously defined by MLVA in the international *Legionella* database whereas VACC11 was described for the first time in this study. The same clusters were observed when the reduced MLVA-8 genotyping scheme was applied and only minor changes were detected. By using MLVA-8 genotyping the six individual genotypes were enclosed into VACC1 (**Figure S4**). These six isolates, which belonged to two different MLVA-8(12) genotypes, were all sgp8 strains in contrast with the rest of the strains enclosed in VACC1, which were all sgp1. They differed from the rest of isolates contained in VACC1 in the number of repeats observed for VNTR markers Lpms31, Lpms33 and Lpms34. Lpms31 presented 17 repeats in comparison to 4 or 0 in the rest of the profiles of VACC1, and VNTR Lpms33 and Lpms34, presented both only one repeat in contrast to 4 and 2 repeats found in VACC1, respectively. VACC1 was the largest cluster including 110 isolates (61.2%). VACC11, VACC2 and VACC5 were in comparison small clusters counting with 31 (17.2%), 19 (10.5%) and 14 (7.7%) isolates respectively. As mentioned above, all sgp1 isolates belonged to VACC1 when MLVA-8(12) was applied. The other clonal complexes (VACC5 and VACC11) contained exclusively non-sgp1 isolates. VACC2 contained mostly non-sgp1 isolates, only two sgp1

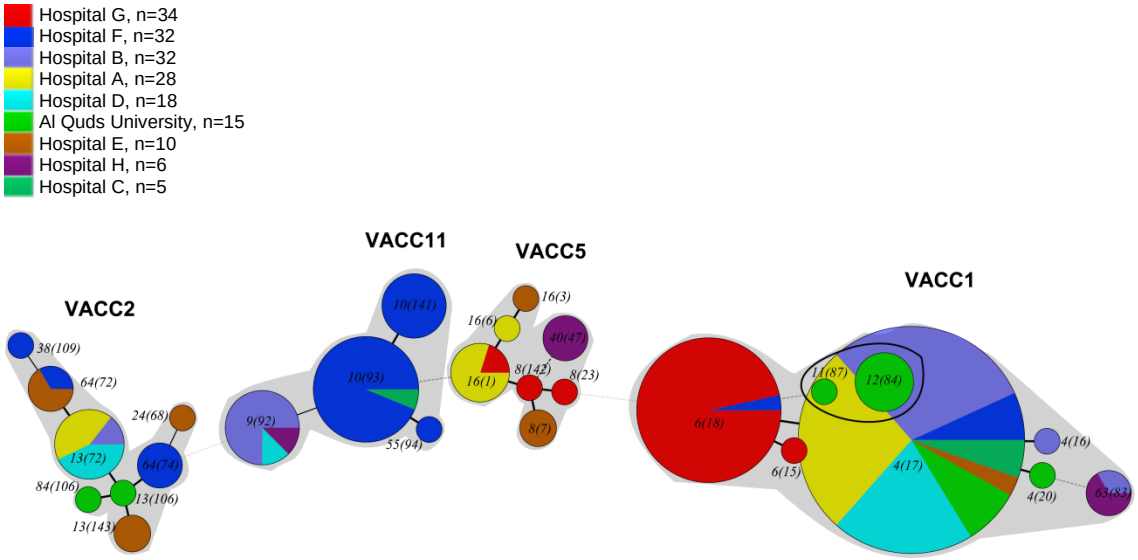
isolates (A100 and A144) isolated from biofilm in different hospitals were included in this clonal complex.

Table 2. MLVA-8(12) genotypes of 180 environmental *L. pneumophila* isolates from the West Bank and the corresponding ST of relevant genotypes.

ST	MLVA-8(12)	No. of MLVA-8(12) genotypes	Frequency (%)
1	Gt4(17)	74	41.1
1	Gt6(18)	30	16.7
461	Gt10(93)	16	8.9
1326	Gt13(72)	10	5.6
461	Gt9(92)	8	4.4
	Gt10(141)	6	3.3
1358	Gt12(84)	5	2.8
1438	Gt16(1)	5	2.8
	Gt40(47)	3	1.7
	Gt63(83)	3	1.7
	Gt64(74)	3	1.7
	Gt13(143)	2	1.1
1482	Gt8(7)	2	1.1
1358	Gt11(87)	1	0.6
	Gt13(106)	1	0.6
	Gt16(3)	1	0.6
	Gt16(6)	1	0.6
	Gt24(68)	1	0.6
	Gt38(109)	1	0.6
	Gt4(16)	1	0.6
	Gt4(20)	1	0.6
	Gt55(94)	1	0.6
	Gt6(15)	1	0.6
	Gt8(142)	1	0.6
	Gt8(23)	1	0.6
187	Gt84(106)	1	0.6
Total		*180*	*100*

The 10% of the total number of isolates (n=18) were sequence typed (**Table 2**). Among the STs observed, ST461 and ST1 were the most frequent (n=5 and n=4 isolates respectively), followed by ST1358 (n=3), ST1326 (n=2), ST1482 (n=2), ST187 (n=1) and ST1438 (n=1). With exception of ST1, a highly abundant sequence type worldwide distributed, most of these sequence types were rarely reported in the *Legionella* database. Environmental and clinical isolates of ST1326 and ST1358 have been found extensively across Europe besides Canada and Russia, both characterized as sgp8 and sgp10. ST461 included environmental and clinical sgp6 strains isolated in Europe, Japan and Australia. By contrast, ST1438 was reported only once in the database, as an environmental sgp6 *L. pneumophila* isolate obtained in Israel. ST1482 was described for first time during this study.

Figure 1. Minimum-spanning tree based on MLVA-8(12) profiles of 180 *L. pneumophila* strains isolated in West Bank. Each circle in the tree represents a different MLVA-8(12) genotype. The genotype designation is indicated within or near the circle, whose size is proportional to the genotype frequency. Different colours in the pie charts refer to the sampling locations (see legend). Thickness of the branches represents the number of different loci. MLVA clonal complexes (VACC) have been shaded. The circles representing the six sgp6 singletons from Al- Quds University (Gt11(87) and Gt12(84)) overlap visually with the circle that represent Gt4(17) due to the high abundance of this genotype. Singletones have been circled to be better distinguished.

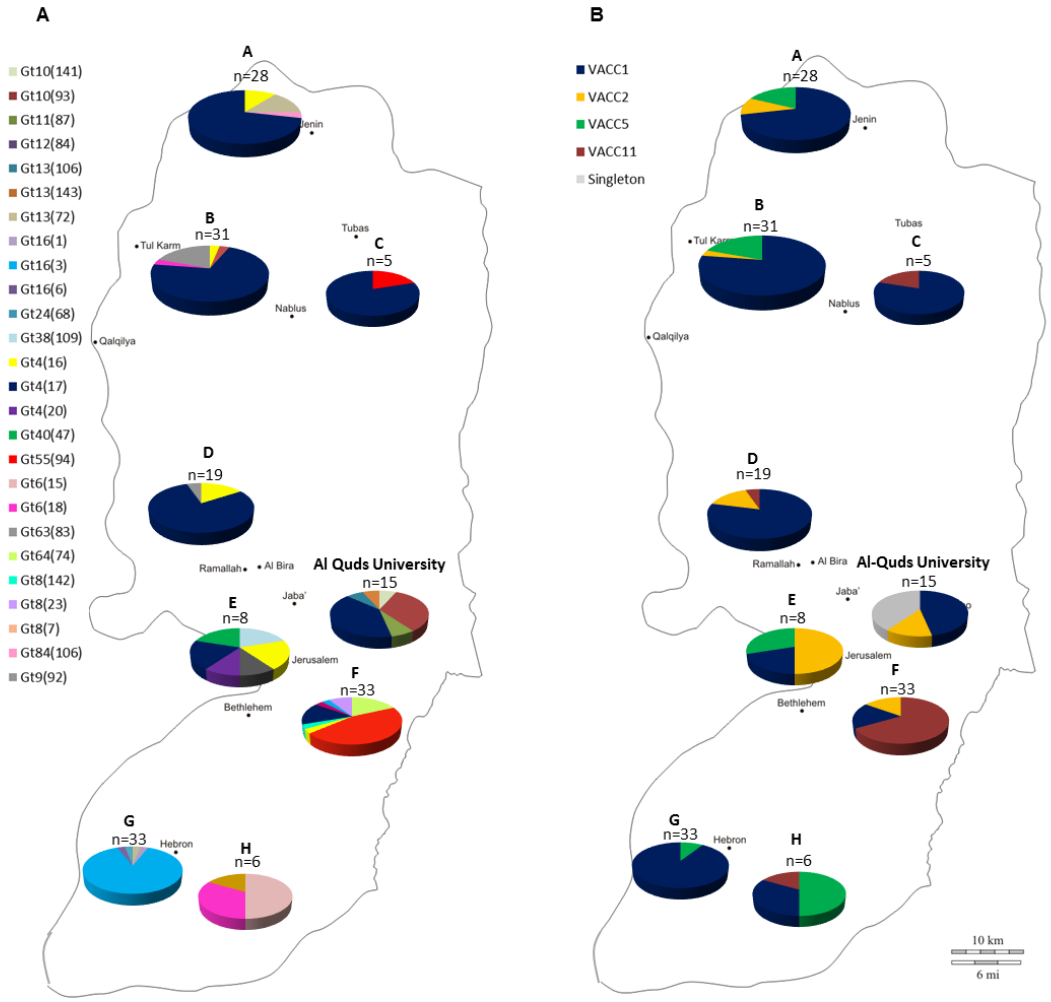


3.4.2 Geographical distribution of MLVA genotypes of *L. pneumophila* in the West Bank

At genotype level, only eight out of the 26 MLVA-8(12) genotypes were isolated in more than one location. The rest 18 MLVA-8(12) genotypes were isolated exclusively in a certain site (**Figure 2**). Gt4(17), was the only genotype present in all hospitals as well as at the University campus. Furthermore, it was not only present but represented a high fraction of the isolates in several hospitals: Gt4(17) was the most abundant genotype in B (n=22, 68.7%), A (n=20, 71.4%), D (n=15, 83.4%), and C (n=4, 80%) hospitals. At the Al-Quds University it accounted for the 40% of the genotypes (n=6). Gt6(18), which only differed in one repeat in the VNTR Lpms35 and also corresponded to ST1, was found at the two close F and G hospitals. However, while in F hospital it was isolated only once, it was the most abundant genotype at G hospital (n=30, 90.9%). The same phenomenon was observed in the case of genotype Gt10(93), which corresponded to ST461. It was found in C and F hospitals, yet, it was isolated only once in C i and in contrast was the most abundant genotype in F (n=15, 45.5%). Other genotypes that were found in more than one location, usually only in one to two locations, can be observed in **Figure 1** and **Figure 2.**

Generally, the most frequent genotypes in each hospital were isolated repeatedly during samplings performed in recurrent years. Gt4(17) was recurrently isolated in A, B, C, D and F hospitals between 2012 and 2014. Gt6(18) was isolated in G hospital in 2013 and 2014. Genotypes Gt10(141) and Gt10(93) were isolated in F hospital in 2012, 2013 and 2014 and Gt9(92) was isolated in B hospital in 2012, 2013 and 2014.

Figure 2. Geographical distribution of **A**) MLVA-8(12) genotypes and **B**) clonal complexes along the West Bank. The number of *L. pneumophila* isolates and letters indicating the designation of the hospitals are representaed above the pie chartses. Dark blue in the geographical representation of genotypes and clonal complexes corresponds to Gt4(17) (see legend), which is the most abundant genotype of VACC1.



At least two distinct clonal complexes were present at each hospital as well as at the Al-Quds University. VACC1, the largest clonal complex was present all across the West Bank. Genotypes belonging to it were isolated at all seven hospitals and at the Al-Quds University. VACC2 isolates were obtained in four hospitals distributed along the West Bank and Al-Quds

University. VACC11, although it was present at five hospitals, it was the major clonal complex at F hospital (n=22, 66.7%). Isolates grouped into VACC5, the smallest clonal complex, were, however, found at four different hospitals located along the Eastern West Bank.

In summary, the majority of the MLVA-8(12) genotypes were found exclusively in one hospital or at the university campus. Only a few MLVA-8(12) genotypes were isolated in more than one location, and these common genotypes were usually much more frequent in one of the locations, with exception of Gt4(17) that was steadily abundant. In some hospitals certain genotypes were especially frequent. When the MLVA-8 profiles were considered, since the diversity did not increase significantly from MLVA-8 (0.772, 95% CI 0.721-0.822) to MLVA-8(12) genotyping (0.790, 95% CI 0.739-0.847), the same general biogeographic patterns were found as when studying the population by using MLVA-8(12).

3.4.3 Genetic diversity of *L. pneumophila* strains from Israel

Overall, 11 clinical and 240 environmental isolates of *L. pneumophila* isolated form 10 different locations and habitats across Northern Israel subjected to MLVA genotyping (**Table S1**). In general, most of the environmental isolates were sgp1 (n=200, 83.3%) in comparison with non-sgp1 (n=31, 12.9%) (**Table 3**). A total of 18 sgp1 isolates were subgrouped by monoclonal antibodies and they were confirmed to belong to the virulence-associated 'MAb3/1-negative' subgroup, characterized by the lack of the virulence-associated epitope. Among the 31 non-sgp1 isolates, the majority were sgp3 (n=26, 84%). All 11 clinical isolates belonged to sgp1.

Table 3. Serogroup and monoclonal antibody subgrouping of 251 environmental and clinical *L. pneumophila* isolates from Israel.

Serogroup	MAb subgroup	Environmental isolates		Clinical isolates	
		No.	Frequency (%)	No.	Frequency (%)
1	OLDA	10	4.2	11	100.0
	Oxford	8	3.3		
	NA[1]	182	75.8		
	Total sgp1	*200*	*83.3*		
3		26	10.8		
2-14 (not 3)		5	2.1		
	Total non-sgp1	*31*	*12.9*		
NA		9	3.7		
NA[1]: Not analysed					

The total set of 251 clinical and environmental isolates was typed in 23 MLVA-8 genotypes (ID=0.643, 95% CI 0.591-0.695), in 34 MLVA-12 genotypes (ID=0.815, 95% CI 0.777-0.852) and 37 MLVA-8(12) genotypes (ID=0.818, 95% CI 0.781-0.855). The majority of MLVA-8(12) genotypes (92%, n=231 isolates) were isolated at least twice, while only 8% of the genotypes (n=20 isolates) were found once (**Table 4**). More than 70% of the isolates (n=184) belonged to only four genotypes. The most frequent isolated genotype was Gt4(17) (n=92, 36.7%), followed by Gt4(16) (n=38, 15.1%), Gt6(18) (n=33, 13.2%) and Gt6(15) (n=21, 8.4%). Gt4(17) and Gt6(18) corresponded, as previously mentioned, to ST1.

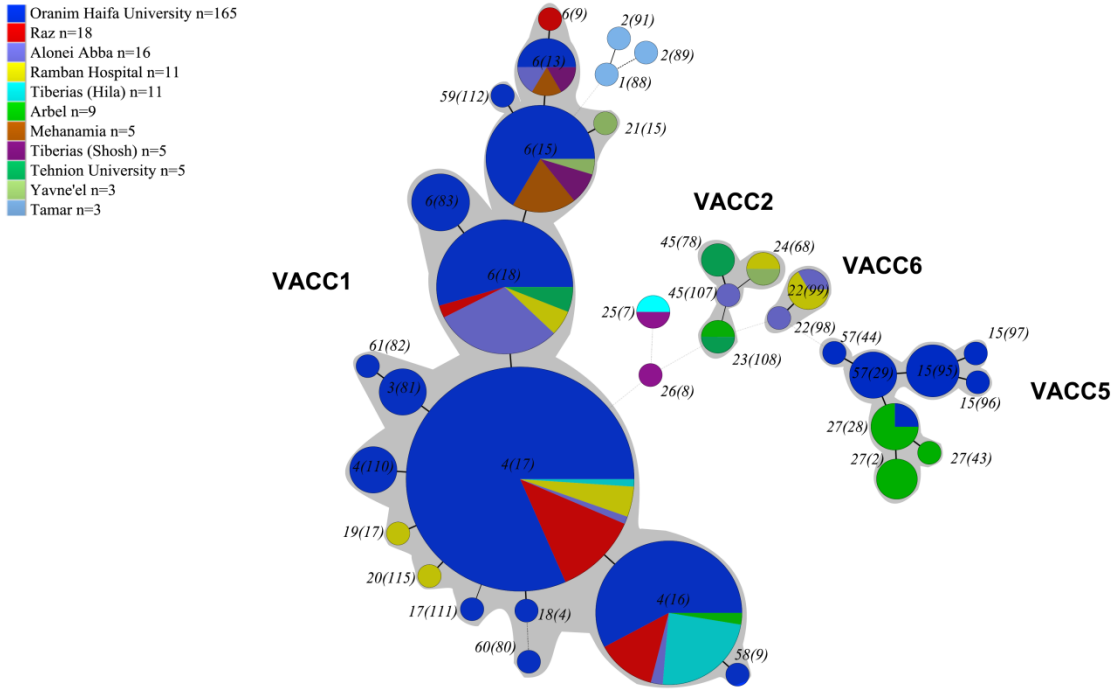
Table 4. MLVA-8(12) genotypes and the corresponding STs of 251 environmental and clinical *L. pneumophila* isolates from the Israel.

ST	MLVA-8(12)	No.	Frequency (%)
1	Gt4(17)	92	36.7
1	Gt4(16)	38	15.1
1	Gt6(18)	33	13.2
1	Gt6(15)	21	8.4
	Gt6(13)	6	2.4
	Gt6(83)	6	2.4
	Gt15(95)	5	2.0
	Gt27(28)	4	1.6
	Gt3(81)	4	1.6
	Gt4(110)	4	1.6
	Gt57(29)	4	1.6
	Gt22(99)	3	1.2
	Gt27(2)	3	1.2
	Gt23(108)	2	0.8
	Gt24(68)	2	0.8
	Gt25(7)	2	0.8
	Gt45(78)	2	0.8
	Gt21(15)	1	0.4
	Gt1(88)	1	0.4
	Gt15(96)	1	0.4
	Gt15(97)	1	0.4
	Gt17(111)	1	0.4
	Gt18(4)	1	0.4
	Gt19(17)	1	0.4
	Gt2(89)	1	0.4
	Gt2(91)	1	0.4
	Gt20(115)	1	0.4
	Gt22(98)	1	0.4
	Gt26(8)	1	0.4
	Gt27(43)	1	0.4
	Gt45(107)	1	0.4
	Gt5(44)	1	0.4
	Gt58(9)	1	0.4
	Gt59(112)	1	0.4
	Gt6(9)	1	0.4
	Gt60(80)	1	0.4
	Gt61(82)	1	0.4
Total		*251*	*100*

At clonal level, 244 of the 251 isolates (97.2%) clustered together in four clonal complexes according to their MLVA-8 and MLVA-8(12) profiles (VACC1, VACC2, VACC5 and VACC6) and only seven isolates remained as single isolates (**Figure S5**). No differences were detected in the formation of the clusters when the different MLVA schemes, MLVA-8 (**Figure S6**) or MLVA-8(12) (**Figure 3**), were used. The clonal complex VACC1 was significantly larger in comparison to the other three clusters and comprised exclusively sgp1 isolates. It enclosed the 85% of the isolates (n=213). VACC2, VACC5 and VACC6 only comprised 2.9% (n=7), 6.8% (n=20) and 1.6% (n=4) of the rest of the isolates, respectively.

VACC6 was as well composed exclusively of sgp1 isolates, while sgp3 isolates predominated in VACC2 and VACC5 clusters.

Figure 3. Minimum-spanning tree based on MLVA-8(12) profiles of 251 clinical and environmental *L. pneumophila* strains isolated in Israel. Each circle represents a different MLVA-8(12) genotype and is proportional to the genotype frequencies. Genotype number is specified within or near the circle. Different colours in the pie charts refer to the sampling locations. Thickness of the branches represents the number of different loci. MLVA clonal complexes (VACC) have been shaded. Clinical isolates are in yellow as isolates from Rambam Hospital (see legend).

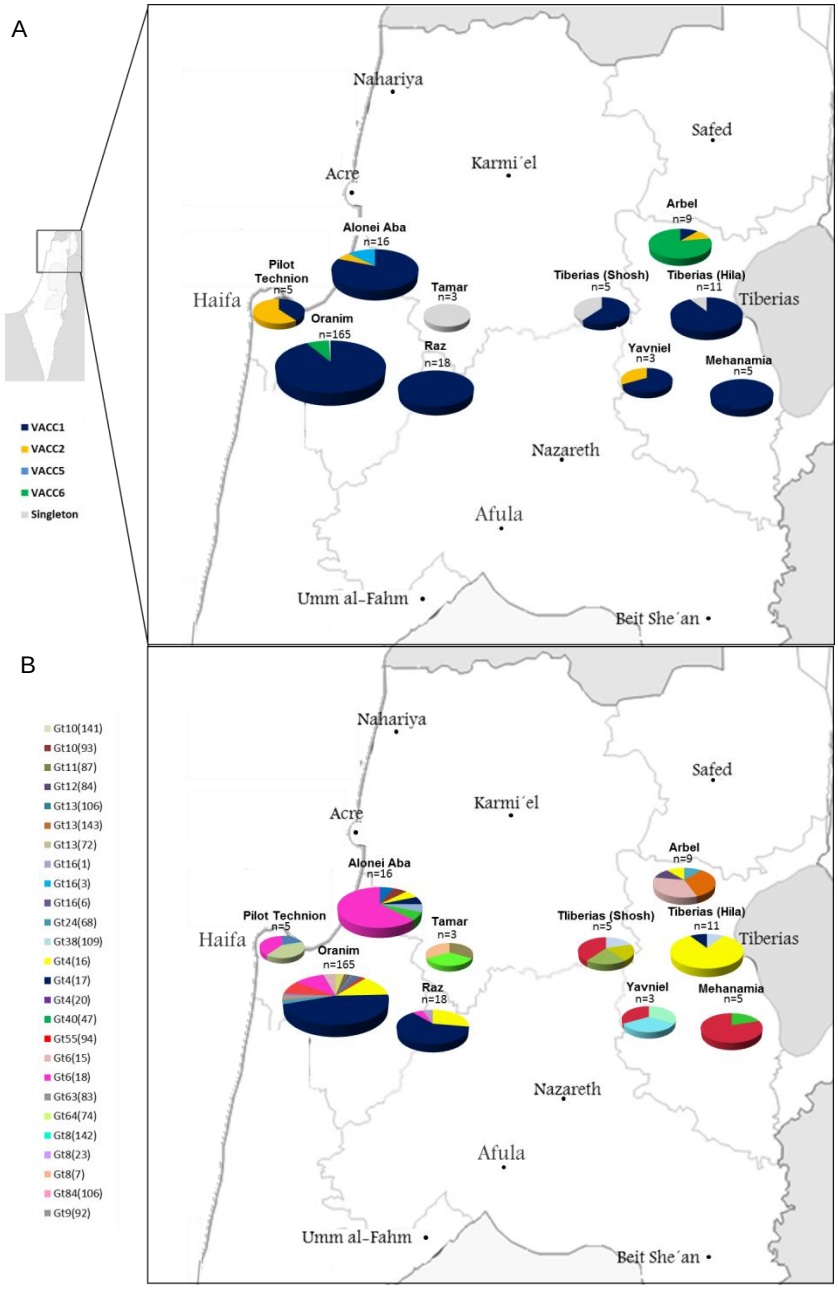


3.4.4 Geographical distribution of MLVA genotypes of *L. pneumophila* in Israel

The study of the population of *L. pneumophila* by using the MLVA-8 genotypes showed the same biogreographic patterns than when MLVA-8(12) genotypes were analyzed, although the resolution of MLVA-8(12) was significantly (ID=0.818, 95% CI 0.781, 0.855) increased respect to MLVA-8 (ID=0.643, 95% CI 0.591, 0.695) and 14 additional genotypes were observed (**Figure 3** and **S6**). In general, the distribution of genotypes was diverse. With exception of the individual genotypes, most of the MLVA-8(12) genotypes where found in more than one location and no clear distribution pattern could be observed. Gt4(17), the most abundant environmental genotype isolated in Northern Israel, was found in five of the ten sampled locations (**Figure 4**). This genotype was most frequent genotype isolated at the Oranim campus in Kyriat Tivon. It was also found in one othe houses sampled as well in Kyriat Tivon and in the near locality of Alonei Abba (**Figure 3** and **4**). In the Eastern part of North Israel, Gt4(17) was isolated from the drinking water system of one of the houses sampled in Tiberias.

The next more frequent genotypes isolated from the environment, Gt6(18), Gt6(15) and Gt4(16) were differenciated of Gt4(17) in only one VNTR locus (Gt6(18) and Gt4(16)) and two VNTR loci (Gt6(15)) (**Figure 3**) and all corresponded to ST1. All these highly genetic related genotypes were, as well as Gt4(17), all frequently isolated at the Oranim campus.

Figure 4. Geographical distribution of clonal complexes (**A**) and MLVA-8(12) (**B**) genotypes in North Israel. Numbers inside the pie charts indicate the number of *L. pneumophila* isolates.

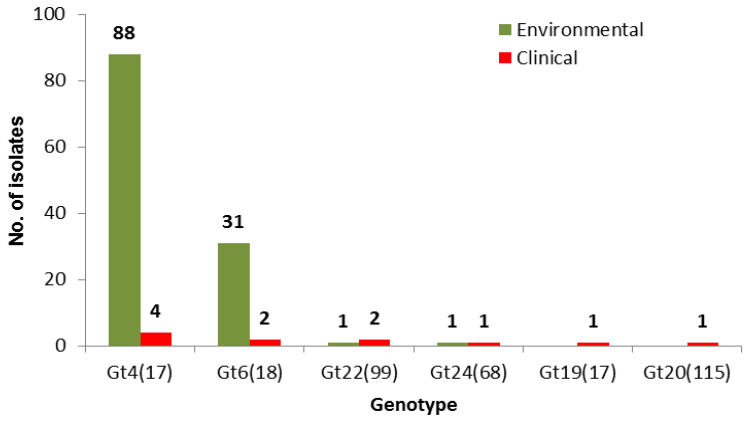


Interesting, Gt6(18) was only found in the Western part of Northern Israel (Oranim and Raz in Kyriat Tivon, Alonei Abba and at the Technion Univeristy located in Haifa) (**Figure S2**). By contrast, Gt6(15) was found, besides at the Oranim campus, in the sampled locations situated in

the Eastern part of the area (Tiberias, Yavne´el and Mehanamia). Gt4(16) was isolated was abundant in both, western and eastern parts of North Israel.

Among these abundant genotypes, Gt4(17) as well as Gt6(18) were also found as clinical isolates. Other two clinical isolates presented genotypes (Gt19(17) and Gt20(115)) with only a single locus different to Gt4(17). Interesting, 9 of the 11 clinical isolates provided by the Rambam Hospital matched their genotype with environmental isolates obtained in North Israel (**Figure 5**). Gt22(99) and Gt24(68) were found in the locations of Alonei Abba and Yavniel respectively, indicating the potential of infection of these environmental strains. Only two genotypes, Gt19(17) and Gt20(115), did not have an environmental match.

Figure 5. MLVA-8(12) genotypes abundances of the 11 clinical isolates of *L. pneumophila* from Israel and its corresponding isolates from the environment.



VACC1, the largest clonal complex, was found in every location sampled in Northern Israel and was the most abundant clonal complex of every location (**Figure 5**). The great majority of isolates in Israel were obtained during a thorough water sampling carried out across the Oranim campus for more than a year (summer 2012 until autumn 2013). A total of 165 *L. pneumophila* isolates were obtained from the seven selected points (points A to G) along the drinking water supply system on the campus (Figure **S1**) More than 92% of the Oranim isolates (n=152) belonged to VACC1 and were characterized as sgp1 (with exception of nine isolates for which no serogroup information was available). VACC1 isolates were found in five out of the seven sampling points (C, D, E, F, G, no *Legionella* was isolated from point B) as exclusive clonal complex in those points. However, most of the isolates obtained in point A (83.4%, n=10) belonged to VACC5 and were subgrouped as sgp3. This chraracteristic spatial distribution of the *L. pneumophila* genotypes along the different sampling points was previously shown by Rodríguez-Martínez et al. (2015) (11) and was confirmed in this study with an increased number of isolates.

Besides the sgp3 isolates obtained in Oranim, VACC5 was as well composed of seven sgp3 isolates obtained in the small locality of Moshav (Arbel sampling point), in the eastern

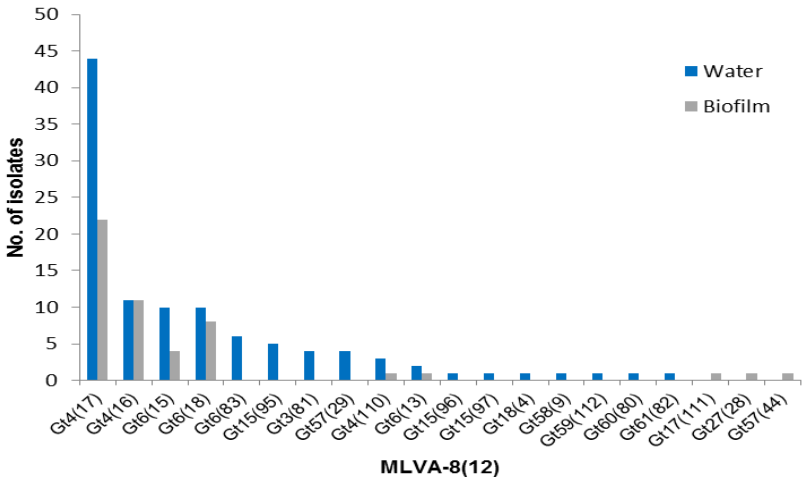
edge close to Lake Tiberias. Isolates belonging to VACC2 had a diverse geographical origin, although it was a small clonal complex. These isolates were found in Haifa (at the Technion University), Alonei Abba and Yavne´el. Furthermore, it contained the genotypes [Gt22(99) and Gt24(68)] of the two clinical isolates from Alonei Abba and Yavne´el. VACC6, formed only by four isolates, was exclusively composed by two clinical isolates and two isolates obtained in the locality of Alonei Abba, situated in the eastern part of North Israel.

3.4.5 Analysis of the MLVA genotypes in different habitats in Israel

The systematic sampling of different habitats (hot water, cold water and biofilm) that was carried out during two years across the Oranim campus (**Figure S2**) and in the rest of selected sampling points in Northern Israel allowed the comparison of diversity of genotypes among the different habitats.

The study by Rodríguez-Martínez et al. 2015 using the MLVA-8 profiles of a set of 68 isolates obtained at the Oranim campus during the first year of sampling showed that the most predominant MLVA-8 genotype in the water system, Gt4, was highly frequent in water as well as in biofilm (58% and 70% abundances, respectively). These results were confirmed for the enlarged dataset of 165 isolates obtained during two years and by the use of the higher resolution MLVA-8(12) genotyping (**Figure 6**). MLVA-8(12) genotypes Gt4(16) and Gt4(17), both corresponding to Gt4 of the MLVA-8 genoytping, were the most abundant genotypes in water (n=55, 51.9%; ID= 0.797, 95% CI 0.729-0.864) as well as in biofilm (n=33, 66%; ID=0.739, 95% CI 0.651-0.827). Likewise, Gt6(15) and Gt6(18), the second more frequent genotypes (corresponding to Gt6 of MLVA-8 genotyping), were abundant in both habitats as well.

Figure 6. MLVA-8(12) ge-notypes abundances of 165 environmental *L. pneumo-phila* strains isolated from water and biofilm across the Oranim campus.



3.4.6 Overview of the clonal structure of *L. pneumophila* isolates in the Middle East and central Europe

In general, the study of the population of *L. pneumophila* isolates in the areas of Northern Israel and along the West Bank showed similar patterns. Sgp1 isolates were more frequent than non-sgp1 isolates in both areas, i.e. 83.3% and 62.3% were characterized as sgp1 in Israel and West Bank, respectively. However, higher diversity was observed among the non-serogroup1 isolates in the West Bank in comparison to Israel. In the sampled locations in Israel, sgp3 prevailed over other non-sgp1 isolates (84% of non-sgp1 isolates were sgp3), which could not be further identified and remained as sgp 2-14. In West Bank, sgp3 was not observed among the group of 54.4% of non-sgp1 isolates that were additionally sub-grouped. In this group the majority of isolates were sgp6, and sgp8 and sgp10 were detected as well.

According to the MLVA genotyping, both areas were characterized by the presence of a few single genotypes, most of their genotypes were isolated more than once. The diversity was slightly higher in Israel (ID=0.818, 95% CI 0.781-0.855) than along the West Bank (ID=0.790, 95% CI 0.739- 0.841), although in West Bank the sampled area was more extent. Nevertheless, the most abundant and more widely distributed genotypes were common to the two areas. In particular, Gt4(17) and Gt6(18), both corresponding to ST1, constituted 51.7% and 58.8% of the total genotypes in Israel and West Bank, respectively (**Figure 7).** At clonal level, five clonal complexes were detected in the two areas (VACC1, VACC2, VACC5, VACC6 and VACC11). VACC1, VACC2 and VACC5 were shared by Israel and the West Bank (**Figure 7**).

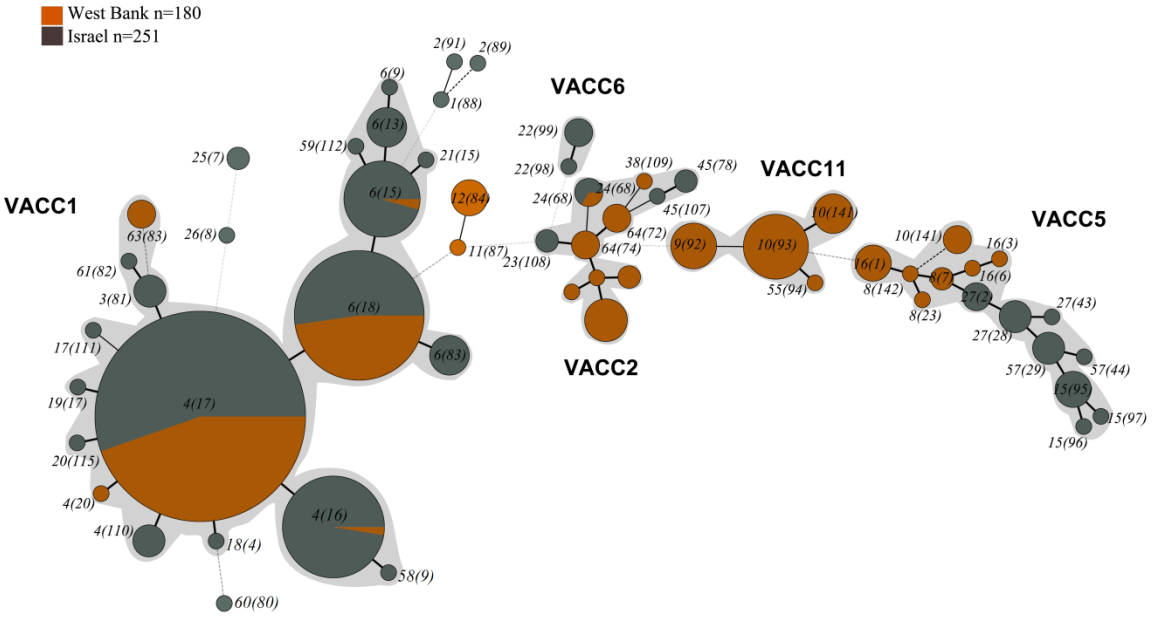
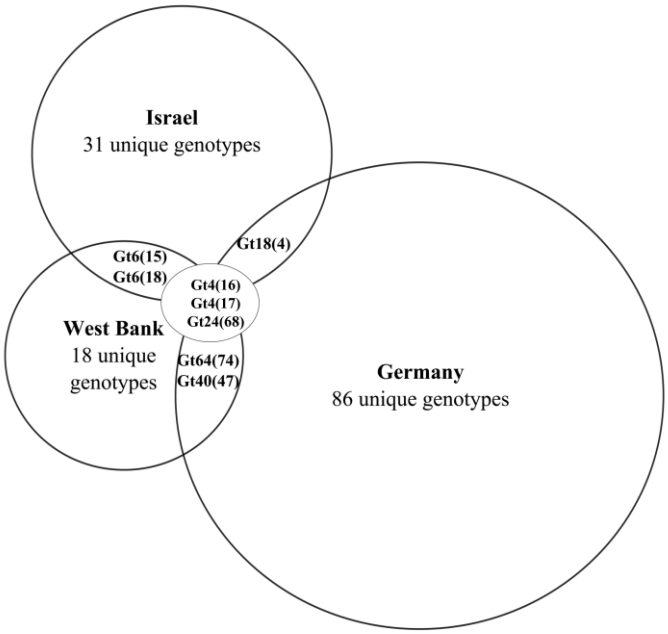


Figure 7. Minimum spanning tree of MLVA-8(12) profiles of 451 clinical and environmental *L. pneumophila* strains isolated in Israel and West Bank. Each circle represents a different MLVA-8(12) genotype and is proportional to the

genotype frequencies. Different colours in the pie charts represent the different geographical areas where the isolates were obtained (see legend). Thickness of the branches represents the number of different loci. MLVA clonal complexes (VACC) are represented by shading.

By contrast, most of the genotypes were found in Germany as single genotypes (71% of the 179 *L. pneumophila* isolates were singletons) and therefore the diversity was higher (ID=0.973, 95% CI 0.962-0.984) than in Israel or the West Bank. However, Gt4(17) was the most common genotype in Germany as well, accounting with 14% of the environmental isolates and 6.6% of clinical isolates. In addition to Gt4(17), only two more MLVA-8(12) genotypes were common between Middle East and Germany, i.e. Gt4(16) and Gt24(68), which corresponded to ST1 and ST93, respectively (**Figure 8**).

Figure 8. Venn diagram of the number of individual MLVA-8(12) genotypes shared among distinct geographic areas. The size of the circle is proportional to the number of genotypes of each area.



Gt24(68) matched to a sgp3 clinical isolate in Germany and to a sgp1 clinical isolate in Israel. In the West Bank this genotype was isolated from biofilm in Al-Makassed Hospital, indicating the presence of pathogenic strains in this location. Germany shared only one MLVA-8(12) genotype with Israel, i.e. Gt18(4), which corresponded as well to ST1, and two genotypes with West Bank, i.e. Gt64(74) and Gt40(47), corresponding to ST440 and ST292, respectively. In spite of the proximity of the two sampling areas in Middle East, only two MLVA-8(12) genotypes were found in common between the West Bank and Northern Israel, i.e. Gt6(15) and Gt6(18), which matched to ST1, in addition to the three genotypes common to Germany. Besides the worldwide distributed ST1, sequence types ST93, ST292 and ST440 had in common their extensive distribution. ST292 corresponds mostly to environmental and clinical, sgp6 isolates observed across Europe and Russia. ST93, has been as well found across Europe

and China and is represented mainly by environmental, sgp3 isolates. Finally, only seven isolates with ST440 are found at the *Legionella* Sequence Based Typing (SBT) database and they were environmental and clinical, sgp6 and sgp10 isolates, obtained from France and Germany. Overall, the majority of MLVA-8(12) genotypes in this study were unique of Europe or Middle East and those common to the two areas were known to be worldwide distributed.

The high exclusivity observed at genotype level between Middle East and Europe contrasted with the high similarity observed at clonal level. Comparing the *L. pneumophila* isolates from the Middle East with the 179 isolates from Germany used in this study and with the international *Legionella* database, it was observed that all five clonal complexes found in the Middle East were found in Europe as well (**Figure 9**).

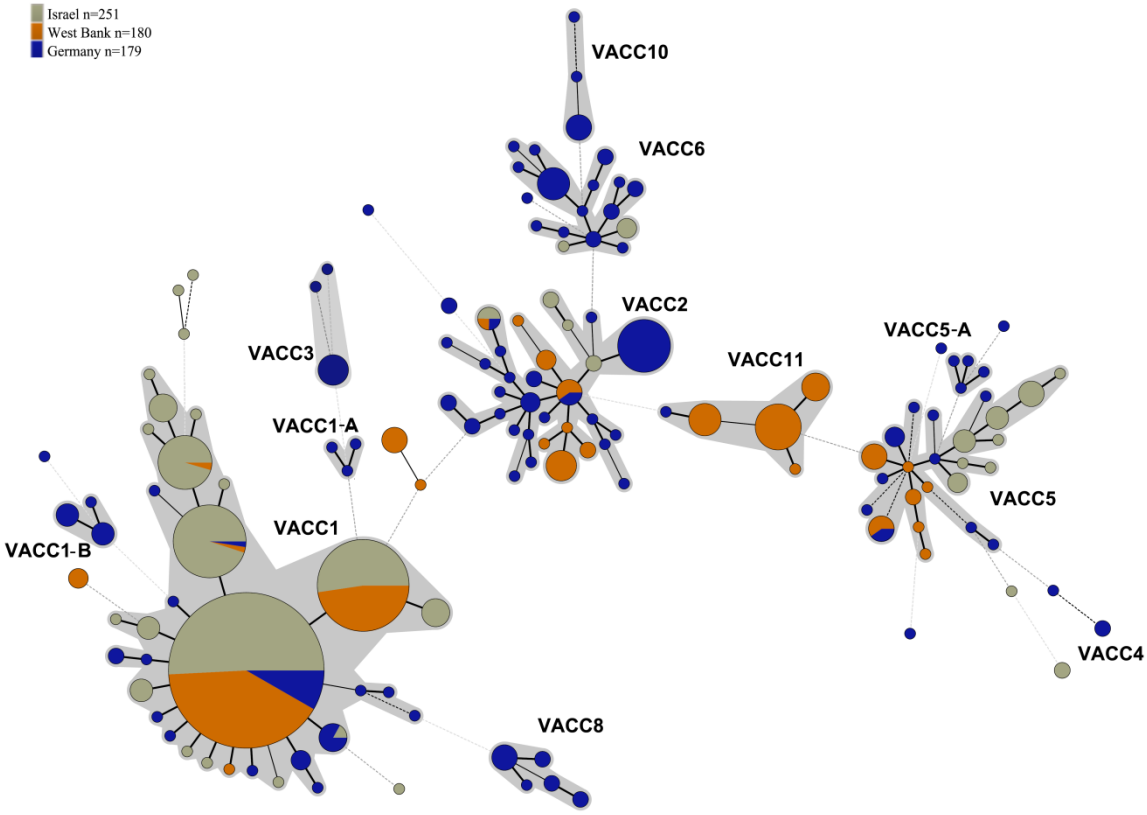


Figure 9. Minimum-spanning tree of MLVA-8(12) profiles of 610 clinical and environmental *L. pneumophila* strains isolated in Israel, West Bank and Germany. Each circle represents a different MLVA-8(12) genotype and is proportional to the genotype frequencies (designation of genotypes not included). Different colours in the pie charts represent the different geographical areas where the isolates were obtained (see legend). Thickness of the branches represents the number of different loci. MLVA clonal complexes (VACC) are represented by shading.

VACC1, the largest clonal complex in the Middle East, was broadly distributed across Europe, as already described in Chapter II. Genotypes that belonged to VACC1 were isolated in distant countries as Greece, Scotland, Sweden and Spain. VACC2 was a large clonal complex in

Germany, observed in all regions. It was observed frequently in France, formed by environmental and clinical isolates of different serogroups. Additionally, several reference strains known to have been originally isolated in United States, among them *L. pneumophila* strain Bloomington-2 and *L. pneumophila* strain Chicago-2, have been included in VACC2. VACC5 was a smaller clonal complex in Germany, although it was widespread. In the database, VACC5 was formed by clinical and environmental isolates of distinct serogroups isolated in different European countries, such as Denmark, Switzerland, Belgium Greece and UK. VACC6 was extensively distributed in Germany and it was composed of clinical and environmental isolates of different serogroups. VACC6 appeared at the *Legionella* database as a large clonal complex formed by numerous environmental sgp1 isolates from France and few clinical isolates isolated from Sweden. VACC11 was not described in the database, so it could be considered as a novel clonal complex found during this study.

A more detailed analysis of the population structure at the level of the VNTR markers that define the genotypes showed that, overall, a higher variability in the number of repeats for most VNTR markers was observed among the isolates obtained in Germany as shown by their HGDI discriminatory index (Hunter Gaston Discriminatory Index). (**Table 5**). This could be due to the general greater diversity of habitats and locations where the isolates have been obtained from. Some VNTR markers appeared to be less variable and showed a reduced number of repeats. This reduction was consistent among the isolates of Middle East and Central Europe, as shown for markers Lpms3, Lpms17 and Lpms40. However, other markers showed a greater variability, and this variability was especially noticeable in the European isolates in contrast to the isolates from the Middle East. Examples of VNTR markers with higher number of repeats in isolates from Europe in comparison to isolates from the Middle East are Lpms13, Lpms31, Lpms35 or Lpms39. In general, the same repeats prevailed independently of the area where the isolate was obtained from. Null alleles were present at different frequencies in distinct VNTR markers of the three areas. Especially high were the frequencies of null alleles in Lpms40 and Lpms44 for isolates from Germany. Remarkably, a new allele of Lpms34 was described during this study. The new allele had a size of 634 base pairs and was formed of four repeats. A total of 31 (17.2%) isolates from West Bank contained this allele. However, it was very infrequently found out of the West Bank area. It was not present in any of the 250 *L. pneumophila* isolates from Northern Israel and it was observed exclusively in one clinical isolate from Germany (L01-138). This allele was not described in previous MLVA studies for *L. pneumophila* and therefore, should be incorporated in the allele assignment reference table provided at *L. pneumophila* MLVA database for future studies.

Table 5. VNTR characteristics of the *L. pneumophila* strains isolated in Israel, West Bank and Germany (average values based on 611 strains).

VNTR	Israel			West Bank			Germany		
	No. of repeats	**HGDI[1] (CI 95%)**	**Null alleles (%)**	**No. of repeats**	**HGDI (CI 95%)**	**Null alleles (%)**	**No. of repeats**	**HGDI (CI 95%)**	**Null alleles (%)**
Lpms1	5	0.241 (0.171-0.312)	0.8	4	0.528 (0.459-0.596)		5	0.528 (0.476-0.580)	
Lpms3	3	0.266 (0.201-0.330)	0.8	2	0.461 (0.420-0.502)		3	0.486 (0.453-0.519)	0.56
Lpms13	6	0.225 (0.157-0.292)	0.8	5	0.579 (0.506-0.652)		10	0.737 (0.684-0.791)	
Lpms17	2	0.016 (1.000-0.038)		2	0.115 (0.053-0.178)		2	0.033 (1.000-0.070)	
Lpms19	4	0.223 (0.157-0.289)	2.39	2	0.022 (1.000-0.053)	1.11	8	0.405 (0.322-0.488)	6.15
Lpms31	6	0.246 (0.176-0.317)		6	0.576 (0.513-0.639)	1.67	15	0.869 (0.853-0.886)	1.68
Lpms33	3	0.237 (0.169-0.304)		4	0.575 (0.506-0.643)		5	0.683 (0.642-0.724)	
Lpms34	4	0.128 (0.072-0.184)	0.8	4	0.503 (0.429-0.577)	2.22	5	0.565 (0.515-0.615)	1.12
Lpms35	8	0.597 (0.550-0.644)	2.79	6	0.687 (0.641-0.733)	1.67	20	0.858 (0.827-0.889)	
Lpms38	3	0.512 (0.466-0.559)	6.77	3	0.249 (0.168-0.330)	4.44	8	0.3 (0.213-0.387)	8.38
Lpms39	7	0.304 (0.231-0.378)		3	0.509 (0.445-0.574)		12	0.813 (0.785-0.841)	6.7
Lpms40	3	0.225 (0.161-0.289)		3	0.493 (0.444-0.541)	3.33	3	0.6 (0.552-0.648)	20.11
Lpms44	5	0.318 (0.247-0.389)	1.99	3	0.498 (0.463-0.533)		3	0.514 (0.446-0.582)	14.53

[1] HGDI: Hunter Gaston Discriminatory Index

3.5 Discussion

3.5.1 Diversity of *L. pneumophila* populations

Not so many studies have reported the status of the epidemiology of *L. pneumophila* in the area that comprises Israel and West Bank (8, 10) and the studies realized in the area of

Israel remarked interesting facts that could deserve more attention and further analysis, as the high frequency of serogroups that have been found just rarely in other areas worldwide or the presence of new sequence types, suggesting the existence of a rich genetic diversity still to be assessed. In the present study, the systematic sampling carried out during two years along a vast area combining the sampling of different types of habitats (bulk water and biofilm), lead to a large collection of 420 environmental *L. pneumophila* isolates that could complement previous studies and bring new insights into the molecular epidemiology and genetic diversity of *L. pneumophila* in this particular area of the Middle East. In general, studies focused on the genetic diversity of *L. pneumophila* in the environment are not so common (23, 24) as those based primarily in clinical isolates (25–27). Studies including environmental samples have been usually realized with the aim of establishing epidemiological links between clinical isolates and their presumed environmental sources during legionellosis outbreaks (21, 28). However, since the *Legionella* infections are originated directly from the environment and do not occur by human-to-human transmission, the study of the genetic diversity and the distributions of *L. pneumophila* strains using this large set of environmental isolates could be relevant in public health control strategies in the Middle East and in later epidemiological studies.

Moran-Gilad et al. (2014) (10) indicated in their study that the tendency of higher number of nosocomial cases of LD in Israel could be due to the under-diagnosis and under-reporting of community-acquired cases. The methodical sampling of water and biofilm from the water systems in different private houses and public buildings in Northern Israel as well as the selection of hospitals distributed along the West Bank allowed the evaluation of the strains that could represent risk of infection, resulting in community-acquired and nosocomial cases of LD. Although most of the *L. pneumophila* isolates from the West Bank (n=177, 98.3%) were obtained from biofilm samples, the strains isolated could be considered representative of the water system since it has been showed that more than 95% of the microbial biomass in a drinking water supply system is found in the biofilms attached to the pipe walls (29) due to the multiple advantages that biofilm represents for microorganisms, as protection of external factors and beneficial interactions with other microorganisms. Additionally, from the point of view of public health biofilm sampling has a great importance since it has been observed that *L pneumophila* strains derived from biofilm replicate significantly more in murine macrophages than planktonic bacteria (30).

Overall, the great majority of strains isolated from the area under study in the Middle East were characterized as sgp1 (83.3% and 62.3% of the isolates in Israel and West Bank, respectively). This serogroup was the most abundant in the area of West Bank as well as in Northern Israel (**Table 1 and 3**). This fact followed the tendency already reported by other studies that have described sgp1 as the most frequently detected environmental serogroup in different geographic regions (5, 31, 32). Besides the high prevalence of sgp1, other serogroups

were isolated, especially along the West Bank, where the fraction of non-sgp1 isolates went up to 37.7%. Sgp6 was particularly abundant (16.7%). Sgp8 and sgp10 were as well isolated in the West Bank, although in smaller proportions (3.3% and 0.7%, respectively). In Israel, the number of non-sgp1 isolates was lower than in the West Bank (16.6%) and sgp3 was the most frequently serogroup observed (10.8%) within this group. This serogroup has been found to be very frequent in drinking water systems in Israel (9, 10). It cannot be excluded that sgp3 was not present among the isolates from West Bank, since only 54.4% of the non-sgp1 isolates could be additionally subgrouped. The results obtained here were highly concordant to those from two studies about the distribution of *L. pneumophila* serogroups in man-made water systems not related to human disease (25, 31). *L. pneumophila* sgp1 was the most frequently isolated serogroup followed by sgp6 and sgp3 in France and the UK, where they were as frequently observed as sgp10. Both sgp3 and sgp6 were the serogroups responsible for more cases of LD after sgp1, according to the European surveillance data (2).

Multi Locus Variable number of tandem Repeats Analysis (MLVA) was the method of choice in this study to evaluate the diversity and population structure of the large set of 431 *L. pneumophila* isolates from the Middle East. Representative isolates were as well typed by Sequence Based Typing (SBT). The combined MLVA-8(12) genotyping scheme showed higher discriminatory power than MLVA-8 in the isolates from Northern Israel and the West Bank. In the West Bank, the number of genotypes increased from 16 to 26 when applying MLVA-8(12) (0.790, 95% CI, 0.739-0.841) in comparison to MLVA-8 (ID=0.771, 95% CI 0.721-0.822). In Israel, the number of genotypes increased from 23 with MLVA-8 genotyping (ID=0.643, 95% CI 0.591-0.695) to 37 with the MLVA-8(12) scheme (ID=0.818, 95% CI 0.781-0.855). Despite the proximity of the two areas, only five MLVA-8(12) genotypes were common between the West Bank and North Israel (**Figure 8**). This could be due to the different origin of the water supplied in both areas, groundwater in West Bank and surface water in Northern Israel. Of the five common genotypes, two of them, Gt4(17) and Gt6(18), were the most frequent and more broadly distributed genotypes in each area **(Figure 1** and **3)**. Furthermore, they were equally abundant in water and biofilm in the drinking water supply systems in Israel and corresponded to ST1. These results confirmed those by Moran-Gilad et al. (2014) (10) where ST1 was described as the most prevalent sequence type in Israel. Interesting, in Israel most of the MLVA-8(12) genotypes were found in more than one sampling location, yet in the West Bank each hospital presented particular genotypes. As demonstrated by Rodriguez-Martínez et al. (2015) (11) water physicochemical parameters, such as temperature and pH, can affect the presence and abundance of particular genotypes in Northern Israel. Therefore, additional analysis should be carried out to validate the presence of potential ecotypes in the hospitals of West Bank.

The populations of *L. pneumophila* in Germany differed nearly completely to the populations of *L. pneumophila* in the Middle East. It was observed that only three MLVA-8(12) genotypes were shared by the two distinct geographic areas. Gt4(17) was one of the common genotypes and, as in the Middle East, it was very abundant among both environmental and clinical isolates in Germany. Specifically, this genotype was the most abundant among the clinical isolates obtained in Israel and the second most abundant in Germany. ST1, the sequence type matching genotype Gt4(17), is one of the most the most abundant sequence type worldwide, as already discussed in Chapter II (27, 31, 33, 34). The high abundance of ST1 in the environment has been reported in several studies. In Japan, the majority of environmental isolates (29%) were ST1 (36) as well as in South Korea, where ST1 was distributed across all sampled facilities and regions and accounted for 48.1% of the isolates (32). ST1 was the most abundant sequence type among environmental isolates in Canada and it was found ubiquitously across the country (34). In a study conducted across the United States, ST1 was the most frequent sequence type among both clinical sporadic and environmental isolates, accounting for the 25% and 49% of the total number of isolates respectively (27). In Europe, ST1 has been also reported the most predominant sequence type among environmental isolates in Germany (37), England and Wales (31), Portugal (38) and Spain (39). ST1, has been found often in Europe very frequently not only in the environment but also responsible of cases of pneumonia (40, 41). Moran-Gilad et al. (2014) (10) showed as well that the 42.8% of the clinical strains from Israel analysed in his study belonged to ST1.

Despite the low number of isolates from this study selected for Sequence Based Typing (10% of isolates from West Bank and 2.4% of isolates from Israel), a novel sequence, ST1482, was identified. Among other unique sequence types that have been observed, ST1438 had been previously identified exclusively in Israel (10) and the rest appeared to be, as ST1, broadly distributed according to the SBT database. For instance, ST1358 was among the most frequently isolated sequence types across a Spanish region (39) and ST461 was a sgp6 strain isolated from different hospitals in Poland (42).

The clonal population structure of *L. pneumophila* in the Middle East was characterized by the presence of five clonal complexes or VACCs (VACC1, VACC2, VACC5, VACC6 and VACC11). VACC11 was first defined during this study and it was formed exclusively by a group of 31 strains isolated from several hospitals in West Bank. VACC1, which main genotype was Gt4(17) and also included the pathogenic *L. pn.* Paris reference strain, was the largest cluster (**Figure 7**) in the Middle East. This cluster enclosed closely related sgp1 strains isolated in most of the sampling locations in Northern Israel and the West Bank. The rest of the clonal complexes were significantly reduced in comparison to VACC1. Especially small was VACC6, which included two clinical isolates and two environmental isolates obtained in the locality of Alonei Abba in Northern Israel. When comparing the population of *L. pneumophila* in Middle

with the population in Germany, it was revealed that VACC1 was the dominant cluster followed by VACC2, which included the *L. pn*. Philadelphia reference strain. This pattern was consistent with previous studies (18). In the study by Visca et al. (2011), the population structure of a set of *L. pneumophila* strains with different origins from the European Union Legionella (EUL) collection was investigated by MLVA-8 genotyping. The 86% of the strains were grouped into clonal complexes, of which VACC1 was the largest cluster followed by VACC2.

This general clonal structure observed in Europe and Middle East with prevalence of VACC1 contrasted with the study at local scale carried out in the French city of Rennes (21). In this study was observed that the whole water supply system of the city was colonized by few closely related strains belonging to VACC6. Two of the other clonal complexes found in the water system of the city, i.e. VACC1 and VACC2, were significantly smaller clusters. It should be pointed out that all VACC6 strains had been isolated from hot water supplies, while VACC1 and VACC2 strains were isolated from cooling towers distributed around the city. Considering the global distribution of single strains and its genetically similar variants, i.e. Gt4(17) and VACC1, it can be suggested that geographical barriers are not the main force influencing the differentiation of *L. pneumophila* strains. However, it cannot be excluded the significance of the geography. As shown in this study, each area (Northern Israel, the West Bank and Germany) was supplied by different water sources and presented mostly unique genotypes. In the other hand, both the existence of global and local strains, suggested that the environmental conditions of the habitats, especially temperature, could play a very important role thriving the evolution of *L. pneumophila*. Ecological barriers could be then responsible for the separation of the distinct clones. Clonal complexes widely distributed could have a wider adaptation range than smaller ones. Alternatively, widely distributed clones could be adapted to ecological conditions more common in water systems. The possible adaptation of the clonal complexes to different ecological niches leads to the concept of ecotype (43). Edward et al. (2008) (44) investigated the clonal population of *L. pneumophila* by analysing the sequences of six loci of the SBT scheme in 335 globally distributed clinical and environmental isolates. The presence of large clonal complexes composed of isolates from very distant localizations also suggested the absent of geographic separation and therefore, it was proposed that the clusters would be adapted to specific ecologies, establishing stable ecotypes. Recently, Rodríguez et al. (2015) (11), also suggested the presence of specific ecotypes in the same drinking water system of the Oranim campus as used in this study. Their study revealed the occurrence of different genotypes adapted to different temperature ranges. Particularly, the most abundant genotype, i.e. Gt4, which corresponded to Gt4(17) and dominated the clonal complex VACC1, was better adapted to colder temperatures (20.6°C). Another genotype found in their study, i.e. Gt15, which belonged to a distinct clonal complex (VACC5), was adapted to significantly higher temperatures (45.1°C). This agrees with the observations from the water system in France (21). Further

research would be needed for a better understanding of the ecological features that might drive the differentiation of the *L. pneumophila* populations into genetic clusters.

3.5.2 Effectiveness of the selection of MLVA as principal genotyping method

Bacterial genotyping is an essential tool in molecular epidemiology. Determining the relatedness between bacterial strains and combining with epidemiological information is crucial in order to understand the transmission and evolution of pathogens. In addition, the use of molecular markers has become popular in ecological studies. Typically, genotyping methods are applied to study organisms in their natural habitats and to evaluate the effect of diverse environmental or anthropogenic disturbance and variations.

Whole genome sequencing (WGS) is an increasingly popular technique with great potential in microbial identification and genotyping (45). WGS has also been shown to be suitable for population and epidemiological studies of different bacteria (46–48). Although further improvements in data analysis and standardization are needed, this method it is considered as a possible routine procedure for surveillance and outbreak investigations (45). In the case of *L. pneumohila*, WGS has been applied in several studies (28, 49, 50), demonstrating its potential in providing high quality typing and epidemiological data. Despite of the advantages associated to WGS, there are situations in which the application of alternative, faster and lower cost genotyping tools are desirable. In this study, the high number of isolates (more than 700 isolates) of *L. pneumophila* collected during extensive sampling campaigns in the Middle East and Germany required a genotyping method able to characterize such large number of isolates in a time and cost efficient manner. Multi Locus variable number of tandem repeats (VNTR) Analysis (MLVA) had been previously described as a high throughput tool for subtyping of different bacterial pathogens (16, 51). MLVA relies on the use of genetic markers as VNTR loci, which are genomic regions with high mutational rate and thus, high number of possible allelic states (17). The capability of multiplexing the amplification of VNTR loci made the approach very rapid. Additionally, the application of capillary electrophoresis increased the throughput, accuracy and the reproducibility of the method. Due to the use of highly variable genetic regions, MLVA has been shown to have high discriminatory power (52, 53). Here, all MLVA schemes combining different number of VNTR loci tested (MLVA-8, MLVA-12 and the joint MLVA-8(12) scheme) revealed higher resolution than Sequence Based Typing (SBT), the standard typing method for *L. pneumophila*. However, concordance with SBT was excellent, making MLVA genotyping method appropriate for the characterization of numerous *L. pneumophila* isolates. Finally, the study of the high amount of isolates from different habitats and geographies by MLVA genotyping presented in this study has provided an effective basis for further research. For instance, further genome analyses of representative MLVA genotypes

can be applied to gain additional insights in the evolutionary forces that shape and form the distinct clonal complexes or lineages. Moreover, physiological and ecological studies of different MLVA genotypes are foreseen to investigate the role of the ecotypes in water supply systems (11).

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3.7 Supplementary material

Table S1. Sampling locations and number of isolates obtained in each location in West Bank and Northern Israel.

Area	Sampling location	Coordenates	No. of isolates	Frequency (%)
West Bank	A hospital	32°27'42"N, 35°17'33"E	28	15.6
	B hospital	32°13'31"N, 35°14'28"E	31	17.2
	C hospital	32°13'22"N, 35°15'43"E	5	2.8
	D hospital	31°53'45"N, 35°12'23"E	19	10.6
	E hospital	31°46'50"N, 35°14'46"E	10	5.6
	F hospital	31°42'39"N, 35°11'52"E	33	18.3
	G hospital	31°33'23"N, 35° 4'59"E	33	18.4
	H hospital	31°31'49"N, 35° 5'59"E	6	2.3
	Al-Quds University	31°45'18"N, 35°15'37"E	15	8.3
Total			*180*	*100*
North Israel	Alonei Abba	32°43'51"N, 35°10'19"E	16	6.4
	Arbel	32°48'73"N, 35°29'80"E	9	3.6
	Mehanamia	32°39'90"N, 35°33'30"E	5	2.0
	Kyriat Tivon (Oranim campus)	32°71'29"N, 35°10'97"E	165	65.7
	Rambam Hospital	32°49'58"N, 34°59'8"E	11	4.4
	Kyriat Tivon (Raz)	32°42'58"N,35°7'39"E	18	7.2
	Kyriat Tivon (Tamar)	32°42'58"N,35°7'39"E	3	1.2
	Technion University	32°46'36"N, 35° 1'23"E	5	2.0
	Tiberias (Hila)	32°47'34"N, 35°30'92"E	11	4.4
	Tiberias (Shosh)	32°47'66"N, 35°31'90"E	5	2.0
	Yavne'el	32°42'31"N, 35°30'13"E	3	1.2
Total			*251*	*100*

Figure S1: Drinking water system sampling map at Oranim campus, Kiryat Tivon, Israel**.** Seven sampling points were selected in order to cover the drinking water system route. Sampling points are marked with capital letters. Points where cold and warm water was sampled are underlined. **A**- Shower. **B**- Garden irrigation faucet. **C**- Sink faucet. **D**- Toilet sink faucet. **E**- Shower. **F**- Sink faucet. **G**- Toilet sink faucet.

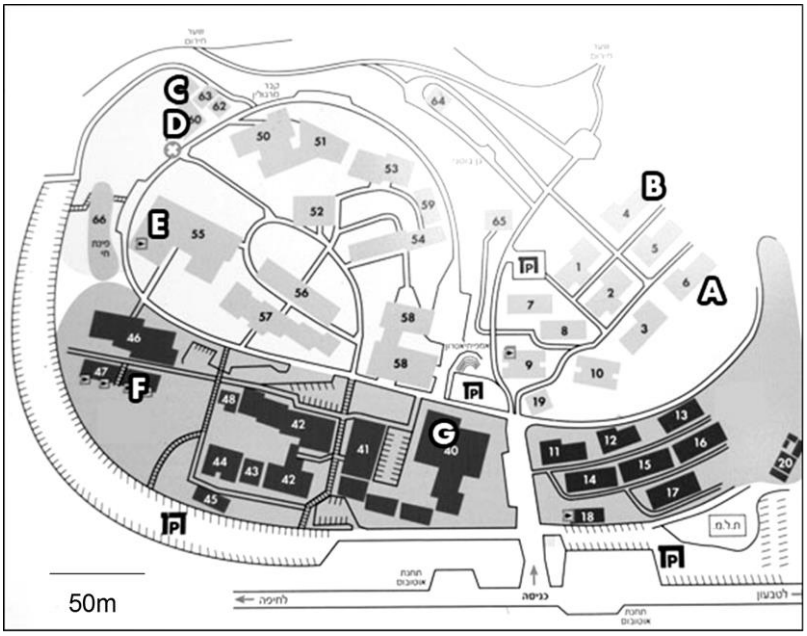
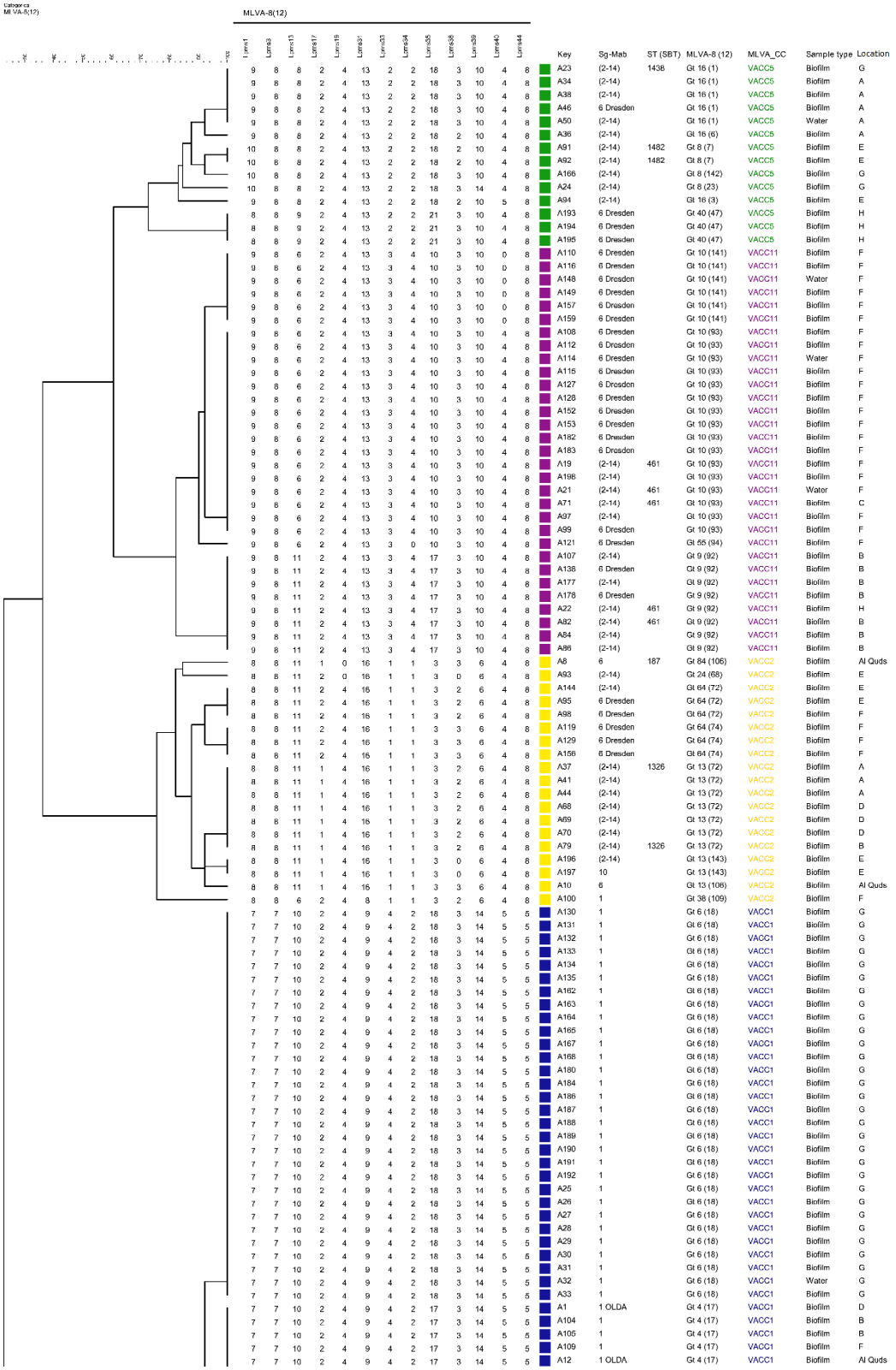


Figure S2. Map of the area under study in Middle East showing the sampling locations in North Israel (blue place marks) and the West Bank (green place marks). Image source: Google earth V 7. 1.5.1557. (December 14, 2015). Middle East. 31°11’42.57’’N, 36°49’33.41’’E, Eye alt 217.22 mi. US Dept of State Geographer. Image Landsat ORION-ME 2016.

Figure S3. UPGMA inferred from the clustering analysis of the MLVA-8(12) profiles of 180 *L. pneumophila* strains isolated from water and biofilm samples taken from the Al-Quds University campus and from seven hospitals along the West Bank. MLVA clusters (VACC) of three or more genotypes were defined using a cutoff of 60% of similarity and are shown with colours.



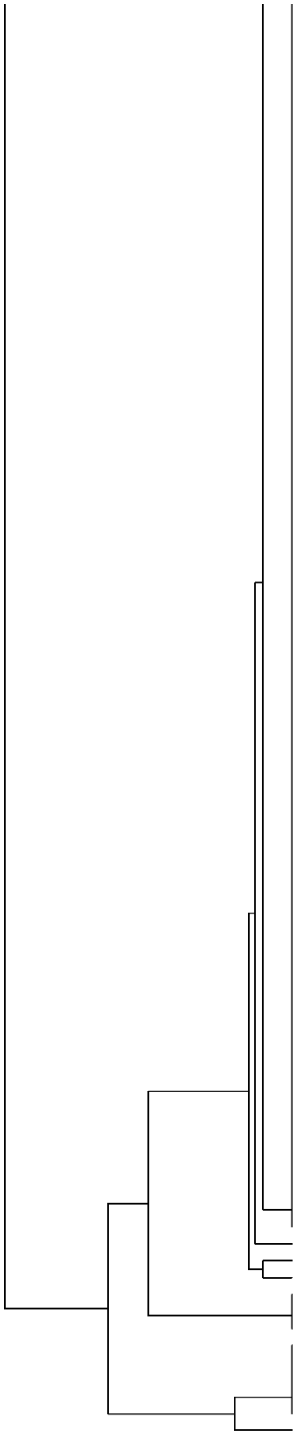


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Figure S4. Minimum-spanning tree based on MLVA-8 profiles of 180 *L. pneumophila* strains isolated in West Bank. Each circle in the tree represents a different MLVA-8 genotype. The genotype designation is indicated within or near the circle, whose size is proportional to the genotype frequency. Different colours in the pie charts refer to the eight sampling locations (see legend). Thickness of the branches represents the number of different loci. MLVA clonal complexes (VACC) have been shaded.

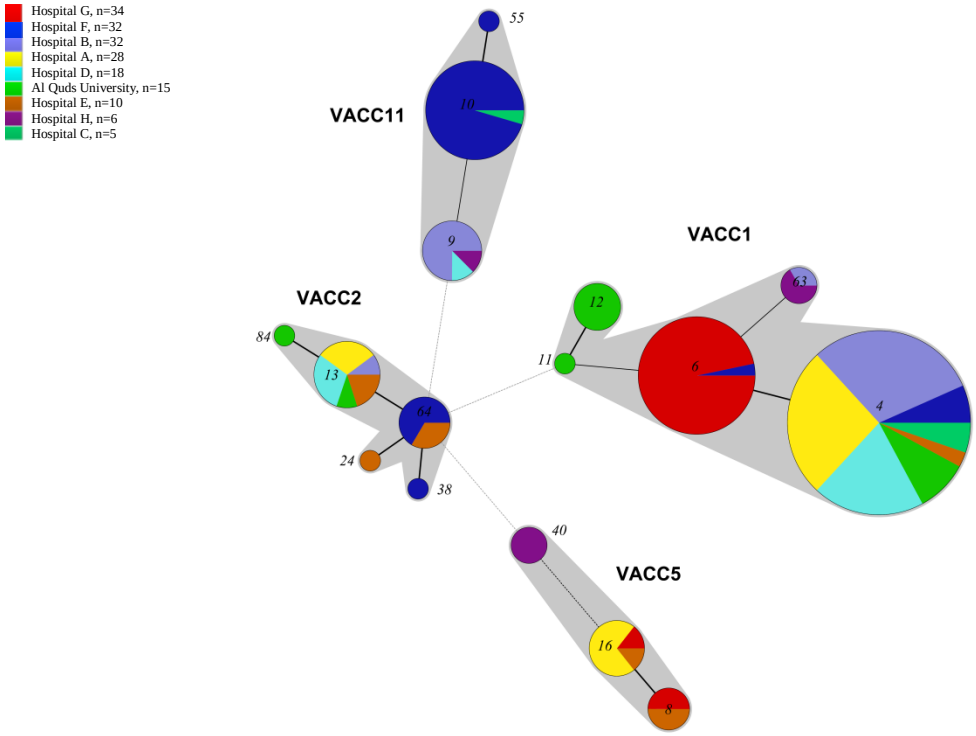
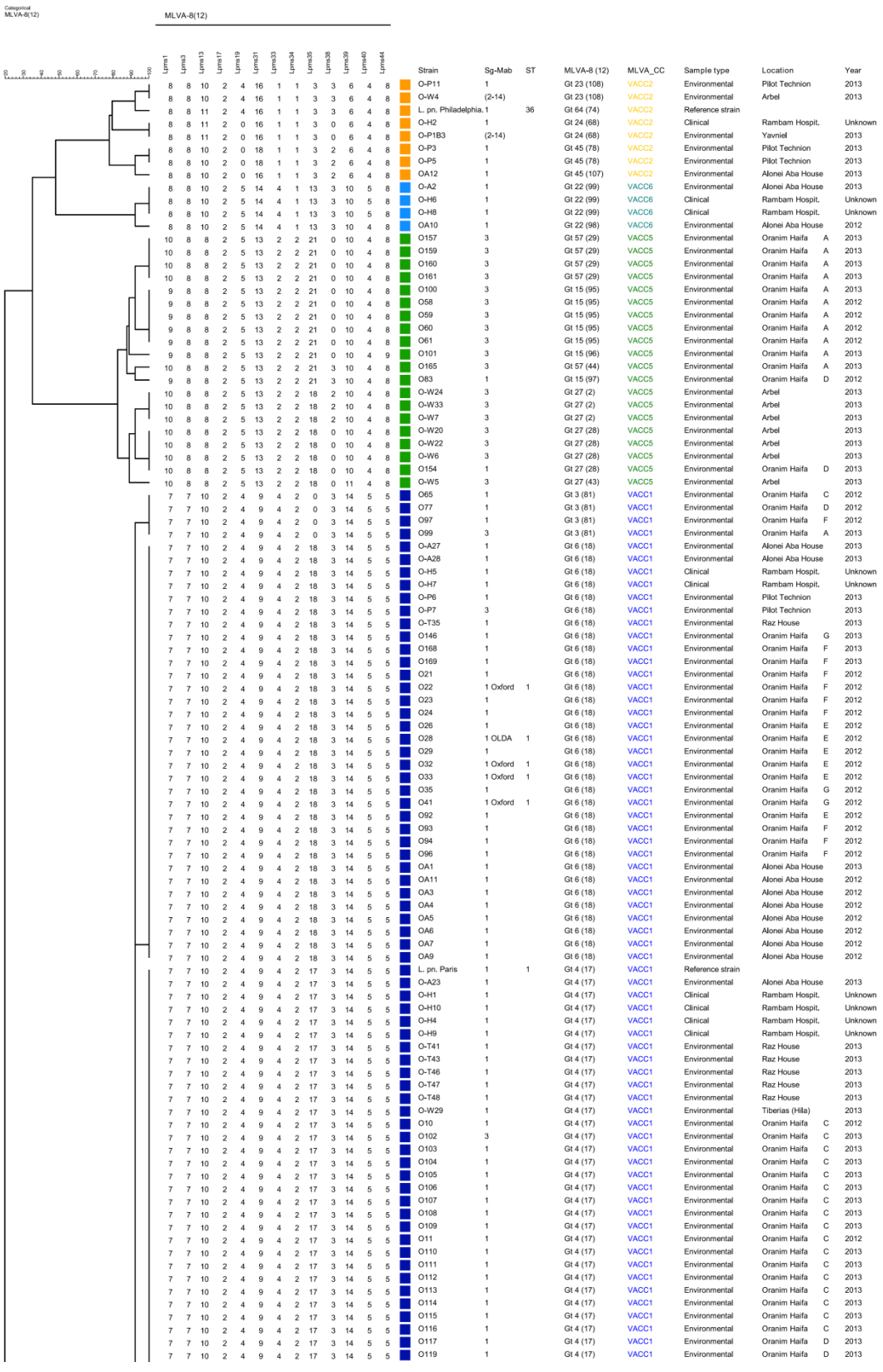
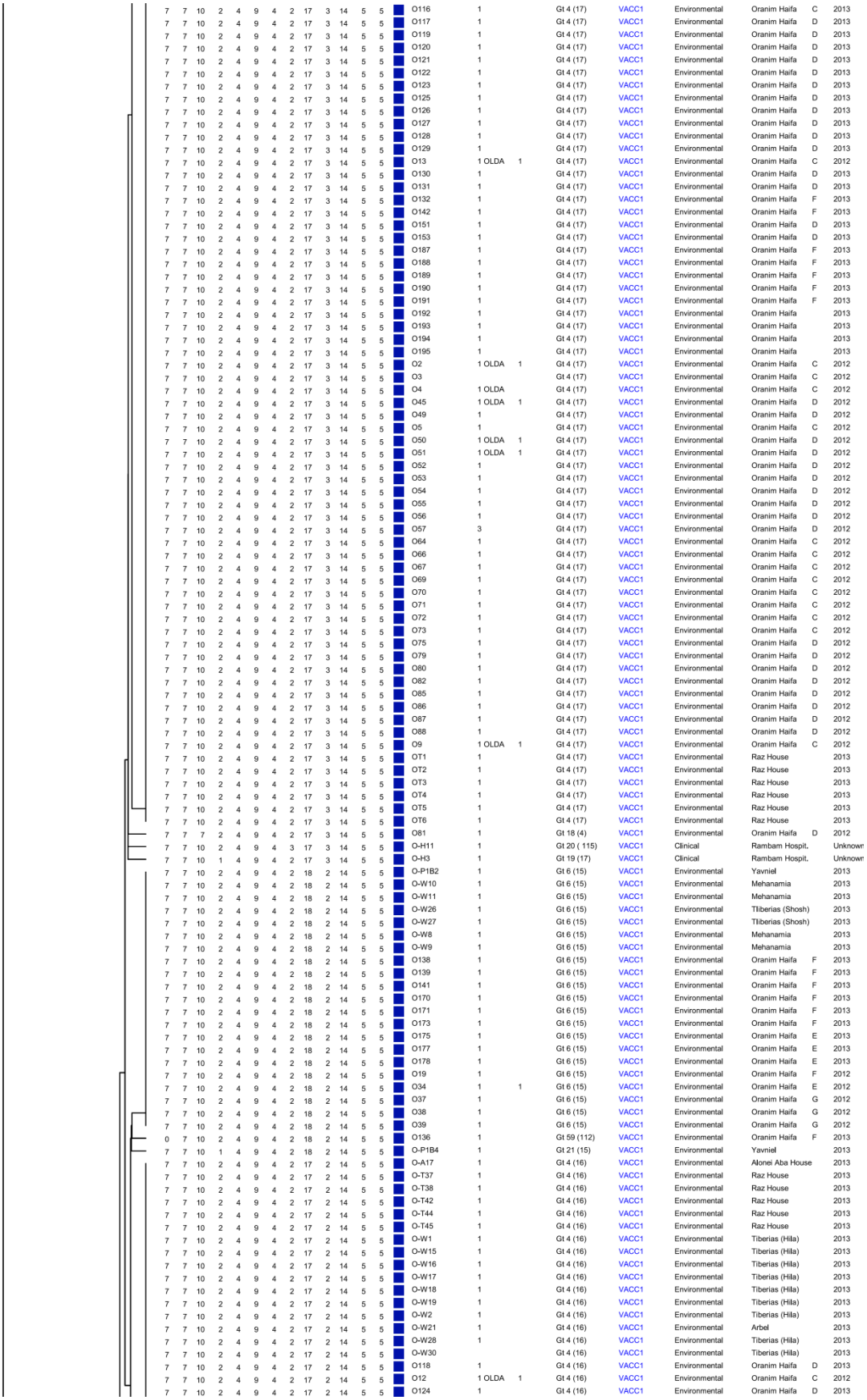


Figure S5. UPGMA obtained from the clustering analysis of the MLVA-8(12) profiles of 251 *L. pneumophila* environmental and clinical strains isolated from nine different locations in North Israel. MLVA clusters (VACC) of three or more genotypes are shown with colors, following the same definition criteria and colour code as the *L. pneumophila* isolates from West Bank (**Figure S3**).





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7	7	10	2	4	9	4	2	17	3	14	5	5	O120	1		Gt 4 (17)	VACC1	Environmental	Oranim Haifa	D	2013
7	7	10	2	4	9	4	2	17	3	14	5	5	O121	1		Gt 4 (17)	VACC1	Environmental	Oranim Haifa	D	2013
7	7	10	2	4	9	4	2	17	3	14	5	5	O122	1		Gt 4 (17)	VACC1	Environmental	Oranim Haifa	D	2013
7	7	10	2	4	9	4	2	17	3	14	5	5	O123	1		Gt 4 (17)	VACC1	Environmental	Oranim Haifa	D	2013
7	7	10	2	4	9	4	2	17	3	14	5	5	O125	1		Gt 4 (17)	VACC1	Environmental	Oranim Haifa	D	2013
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7	7	10	2	4	9	4	2	17	3	14	5	5	OT5	1		Gt 4 (17)	VACC1	Environmental	Raz House		2013
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7	7	10	2	4	9	4	2	17	2	14	5	5	O-W1	1		Gt 4 (16)	VACC1	Environmental	Tiberias (Hila)		2013
7	7	10	2	4	9	4	2	17	2	14	5	5	O-W15	1		Gt 4 (16)	VACC1	Environmental	Tiberias (Hila)		2013
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7	7	10	2	4	9	4	2	17	2	14	5	5	O-W17	1		Gt 4 (16)	VACC1	Environmental	Tiberias (Hila)		2013
7	7	10	2	4	9	4	2	17	2	14	5	5	O-W18	1		Gt 4 (16)	VACC1	Environmental	Tiberias (Hila)		2013
7	7	10	2	4	9	4	2	17	2	14	5	5	O-W19	1		Gt 4 (16)	VACC1	Environmental	Tiberias (Hila)		2013
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7	7	10	2	4	9	4	2	17	2	14	5	5	O-W28	1		Gt 4 (16)	VACC1	Environmental	Tiberias (Hila)		2013
7	7	10	2	4	9	4	2	17	2	14	5	5	O-W30			Gt 4 (16)	VACC1	Environmental	Tiberias (Hila)		2013
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7	7	10	2	4	9	4	2	17	2	14	5	5	O12	1 OLDA	1	Gt 4 (16)	VACC1	Environmental	Oranim Haifa	C	2012
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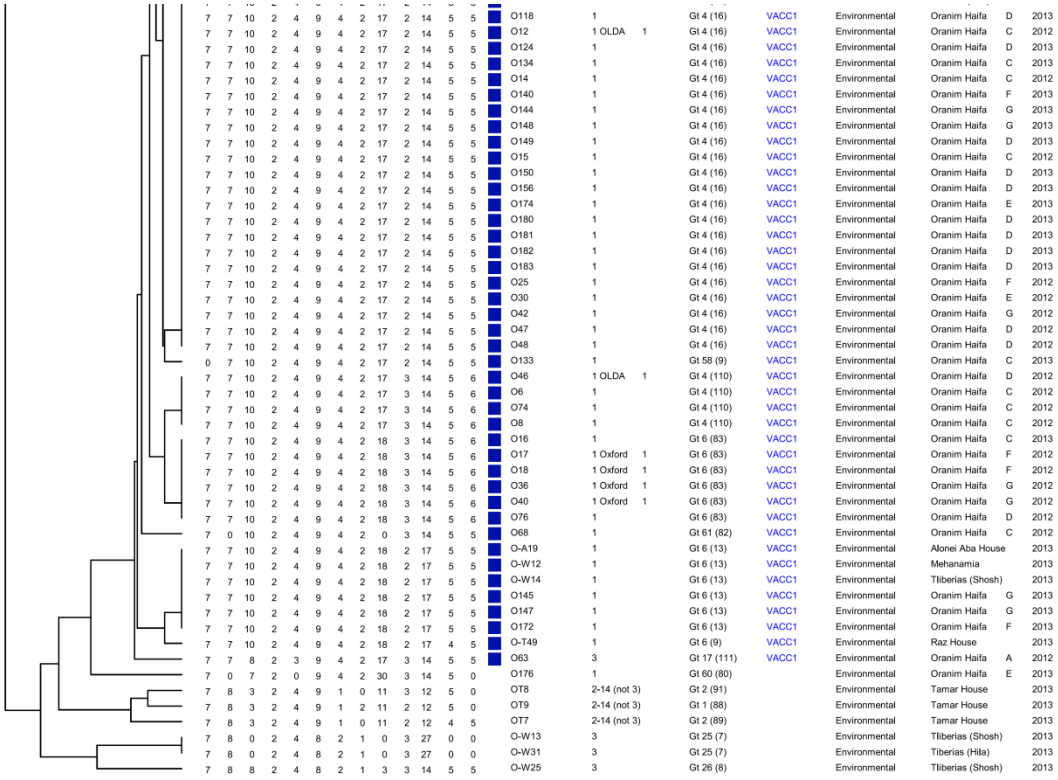
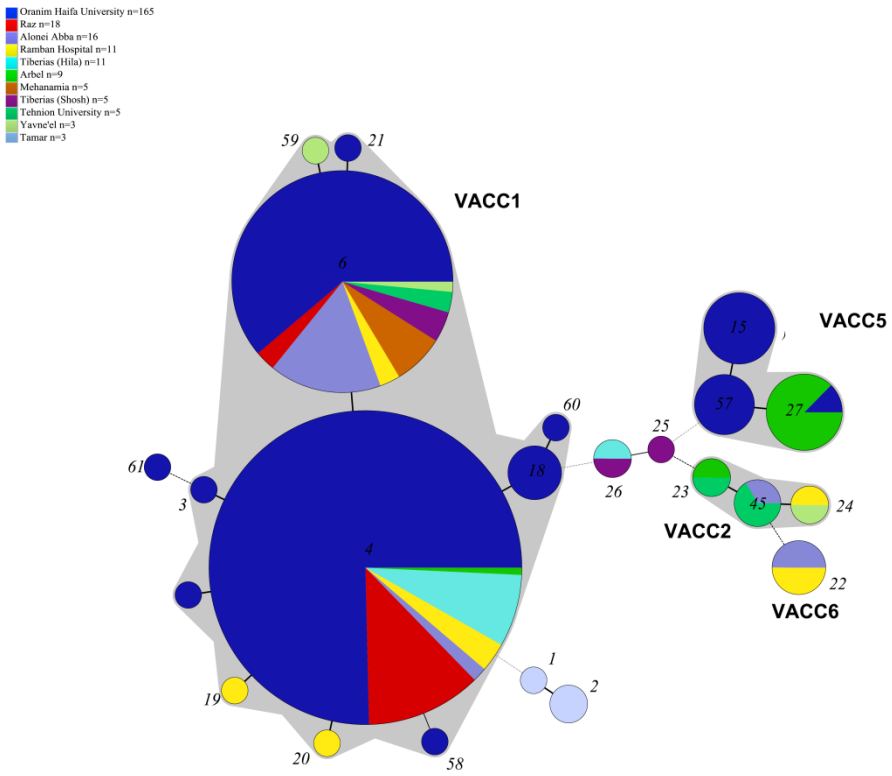


Figure S6. Minimum-spanning tree based on MLVA-8 profiles of 251 clinical and environmental *L. pneumophila* strains isolated in Israel. Each circle represents a different MLVA-8(12) genotype and is proportional to the genotype frequencies. Genotype number is specified within or near the circle. Different colours in the pie charts refer to the sampling locations. Thickness of the branches represents the number of different loci. MLVA clonal complexes (VACC) have been circled. Clinical isolates are indicated as isolates from Rambam Hospital (see legend).



Chapter IV

Assessment of growth and infectivity potential of environmental and clinical isolates of *L. pneumophila* using different experimental approaches

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4.1 Abstract

Legionella pneumophila is an intracellular pathogen of environmental protozoa that inhabits natural freshwater environments as well as drinking water distribution systems (DWSS). *L. pneumophila* is able to cause Legionnaires´ disease due to its capacity to replicate within human macrophages after inhalation of contaminated aerosols. Temperature is one of the key factors affecting the growth of *L. pneumophila* in freshwater systems. *L. pneumophila* virulence is also known to be dependent on growth temperature. In this study, the effect of distinct temperatures (15ºC, 22ºC, 30ºC, 37ºC, 40ºC, 43ºC and 45°C) on growth in liquid medium of 26 clinical and 37 environmental isolates characterized by MLVA genotyping was examined. In addition, the virulence of a total of 85 isolates was tested by in-vitro assays using THP-1 human cell-like macrophages. Results showed that the growth abilities of clinical and environmental isolates differ with temperature. Environmental isolates presented higher ability to grow at low temperatures (15°C, 22°C) while clinical isolates appeared to be more adapted to grow at high temperatures (30°C, 43°C). Clinical and environmental isolates presented their optimum growth temperature at 37ºC and both groups of isolates could not be differentiated at this temperature. Growth temperature also appeared to influence specific genotypes. In particular, the growth of genotype Gt4(17), which corresponded to the globally distributed sequence type ST1, was significantly enhanced at low temperatures. The assessment of infectivity revealed the potential high pathogenicity of certain genotypes and clonal complexes. These findings may assist the understanding of the epidemiology of individual *L. pneumophila* strains and provide insights into the ecology of this opportunistic pathogen.

4.2 Introduction

Legionnaires´disease (LD) is an often fatal pneumonia caused by the ability of *L. pneumophila* strains to replicate within alveolar macrophages. It has been demonstrated that *L. peumophila* has gained this ability by exploiting in the human cells the same conserved signaling pathways as it uses to replicate inside protozoa, its natural hosts (1). Both in protozoa and in human cells, an important virulence factor is the Dot/Icm type IVB translocation system. Currently, it has been established the Dot/Icm type IVB system is responsible in the strain *L. pneumophila* Philadelphia-1 for the translocation of over 330 proteins into the host cell, which represents over 10% of the proteome of this strain (2). Most of these effectors have been shown to have function redundancy; therefore, the lack of some of them does not usually decrease or eliminate the virulence of the strain. Besides the Dot/Icm type IVB translocation system *L. pneumophila* strains possess additional virulence factors common to other bacteria such as lipopolysaccharides (LPS) (3).

The virulence varies thoroughly within different strains of the species *L. pneumophila.* Some strains and serogroups are more virulent than others. Epidemiological studies have shown that of all *Legionella* species, *L. pneumophila* serogroup1 (sgp1) is the most virulent and the most common cause of Legionnaires´ disease (LD) (4). In the same European-wide study, Helbig et al. (2002) (4) indicated that the majority of sgp1 strains presented the virulence epitope and were classified as MAb 3/1 positive (67%) while only the 12% were subtyped as MAb 3/1 negative. Furthermore, different studies in the US and Europe have revealed that most of the cases of LD are due to only few strains. They presented similar results, indicating that some of the sequence types (ST) of clinical and environmental isolates of *L. pneumophila* sgp1 were responsible for outbreaks and sporadic cases in the US and Europe, among which ST1 or ST47 were very frequent (5–7). In addition, it has been demonstrated that the most common clinical STs are rarely found in the environment and vice versa (5, 8). The rapid advances in genome sequencing have revealed new features of the *L. pneumophila* pathogenesis (9). Yet, it is not completely understood what makes certain strains more pathogenic than others.

The ecology of *L. pneumophila* could play an important role since other factors besides virulence, as the bacterial concentration at the source and the success to disseminate through aerosol droplets, can lead to cause infection. The presence of biofilm in freshwater systems and the interaction with their natural host are essential factors that influence the survival and growth of *L. pneumophila* in aquatic environments. In addition, water temperature is crucial in the colonization of drinking water distribution systems (DWSS). Rodríguez-Martínez et al. (2015) (10) have demonstrated the correlation between the temperature in a local drinking water system and the prevalence of different MLVA genotypes. Different genotype distribution patterns were observed at low temperatures (about 20°C) and high temperatures (above 40°C), suggesting the existence of ecotypes adapted to cold or warm freshwater.

In order to get insights into the physiology related with temperature and virulence of *L. pneumophila* strains at both genotype and clonal level, the growth and the infectivity potential were studied. On the one hand, the virulence potential of a large set of strains composed of 85 environmental and clinical isolates characterized by MLVA high resolution genotyping were studied by macrophage infectivity assays. As pointed out by Mercante and Winchel (2015) (11), the relevance of the genotypes for health can only be finally assessed by isolate-based studies in macrophage assays. In this study, the THP-1 human macrophage-like cell line was selected for the infectivity assays due to the extensive use as model for *Legionella* virulence, in transcriptome analysis as well as in the assessment of the virulence of environmental strains (12, 13). The uptake, intracellular replication or the cytotoxicity to host cells were examined. On the other hand, the physiology of 63 strains was evaluated by studying growth at seven different temperatures (15°C, 22°C, 30°C, 37°C, 40°C, 43°C and 45°C) in liquid medium. The evaluation of the growth allowed inferring differences between clinical and environmental strains as well as differences between genotypes.

This study aimed to help understanding the pathogenicity and the role of the temperature in the growth of *L. pneumophila*, since both traits are essential for the ecology of this pathogen. In particular, the examination of these traits at the genotype and clonal level could assist epidemiological studies and the prevention of legionellosis.

4.3 Material and methods

4.3.1 Bacterial strains

A set of 26 clinical and 37 environmental strains of *L. pneumophila* were used in this study to investigate growth in liquid culture at different temperatures. To study the infectivity to human macrophages, the number of strains was increased to 85 (34 clinical and 51 environmental strains) (**Table S1**). Strains were selected according to their MLVA-8(12) genotype and their isolation abundance. The overall strategy of strain selection aimed to have a highly diverse set of strains, with a representation of as many different MLVA genotypes as possible. Therefore, those genotypes isolated at a minimum of three times were considered for analysis. In addition, the selection of the strains also focused on the study of the most abundant genotypes, such as Gt4(17), which has been isolated in different habitats and geographic regions (Europe and the Middle East). Gt4(17) strains from Israel, the West Bank and Germany isolated from water and biofilm were included in this study.

4.3.2 Determination of growth parameters of *L. pneumophila* strains in liquid culture

Growth of 63 *L. pneumophila* strains (26 clinical and 37 environmental strains) (**Table S1)** was determined in BYE liquid medium at six different temperatures: 22°C, 30°C, 37°C, 40°C,

43°C and 45°C. In addition, 34 out of the 63 strains were also studied at 15°C. All *L. pneumophila* strains were plated in BCYE agar plates and incubated at 37°C for three days. To limit the variability in the physiological state of bacteria that will serve as inoculum, all strains were additionally precultured in broth until the bacteria had reached the stationary phase (the time needed for the strains to achieve the stationary phase was previously determined). For that, 50 ml of 1:2 diluted BYE liquid medium [10 g N-(2-Acetamido)-2-aminoethanesulfonic acid (ACES), 10 g yeast extract, 0.4g L-cysteine and 0.25 g ferric nitrate and 1 g α-ketoglutaric acid per litre of sterile water] were inoculate with fresh biomass and incubated at 37°C with agitation (100 rpm) for 48 h. After incubation, precultures were diluted with fresh medium and the optical density at 600 nm (OD_{600nm}) of all the strains were set to of 0.01 (equivalent to cell density of 10^7 cells/ml) to standardize the initial inoculum. Microtiter flat-bottom plates containing 200 µl of culture per well at OD_{600nm} 0.01 were incubated at the specific temperature in a Bioscreen C growth curve analyzer set at continuous shaking. The OD_{600nm} was measured every 15 min for 48 h when studying growth at 30°C, 37°C, 40°C, 43°C and 45°C, for 140 h to study growth at 22ºC and for 400 h at 15ºC. Each strain was studied using five replicates at each temperature. Five replicates of only BYE medium without bacteria were included in each plate to serve as negative control and blank values for OD_{600nm}.

4.3.3 Growth of *L. pneumophila* strains in human macrophage-like cell line

A total of 85 *L. pneumophila* strains (34 clinical and 51 environmental strains) (**Table S1**) were selected to study their pathogenicity**.** Before the infection experiments, *L. pneumophila* strains were grown in 50 ml of BYE liquid medium 1:2 diluted with sterile water for 48 h at 37°C to assure that all strains had reached their stationary phase. The time needed for the strains to reach the stationary phase was previously determined. Bacterial density was assessed by the absorbance at 600 nm with a spectrophotometer Nanocolor Vis (Macherey-Nagel, Düren, Germany). Absorbances were previously calibrated with a standard curve of colony forming units (CFU). Briefly, 10 ml of bacterial culture was centrifuged at 3500 rpm during 15 min, the pellet was suspended in 5 ml of phosphate buffer saline and the optical density at 600nm was adjusted to 0.7, which was equivalent to 10^9 bacteria/ml. The number of cells was checked by plating in BCYE agar plates and counting the CFU. Bacteria was diluted with RPMI-1640 modified medium (Gibco, Germany) to the specific multiplicity of infection (MOI).

THP-1 cells (ATCC TIB-202TM) were maintained in RPMI-1640 modified medium (Gibco, Germany) containing 10% fetal bovine serum (FBS) (Gibco, Germany) and 2mM L-Glutamine (Gibco, Germany) in a humid atmosphere containing 5% CO_2 at 37°C. Before each infection experiment, exponentially growing THP-1 monocytes were washed with complete medium, counted and incubated with phorbol-12-myristate-13-acetate (PMA) (Sigma Aldrich, Germany) at a concentration of 100ng/ml for 48h in 5% CO2 at 37°C to induce maturation of

the monocytes into macrophage-like adherent cells. Adherent cells were washed three times with PBS prior to infection.

Intracellular multiplication assays of *L. pneumophila* strains were carried out as previously described (14). Briefly, differentiated THP-1 macrophages ($2 \cdot 10^5$ cells per well) in 24-well plates were infected in triplicate with 1 ml of bacterial suspension at a multiplicity of infection (MOI) of 1. After 1 h incubation, cells were washed twice with PBS and incubated with 1 ml of RPMI medium containing 100 µg/ml gentamycin (Sigma-Aldrich, Germany) for 1 h at 5 % CO2 and 37°C to kill extracellular bacteria. Infected macrophages were lysed after 1 h, 24 h and 48 h using 1 ml of 0.25% Triton 100X (Sigma-Aldrich, Germany) during 20 sec. Dilutions of the lysate were plated in triplicates in BCYE agar plates and incubated at 37°C to determine the CFU.

Uptake of the different *L. pneumophila* strains into the macrophages was assessed following the procedure of the intracellular multiplication assays described above. However, THP-1 macrophages were infected using a higher MOI for shorter time (MOI 50 for 30 min).

Cytotoxicity assays were carried out by infecting THP-1 macrophages seeded in 96-well plates at a density of 10^5 cells per well with *L. pneumophila* strains at different MOIs (MOI 10, MOI 25, MOI 50 and MOI 75) during 1 h at 37% and 5% CO_2. To measure the percentage of viable macrophages after the infection a colorimetric assay based in the oxidation-reduction of resazurin acid (Alamar blue) (Sigma Aldrich, Germany) was used. After infection and washing with PBS, macrophages were incubated with 200 µl of RPMI medium supplemented with 10% of Alamar blue (Sigma-Aldrich, Germany). Absorbance was measured at wavelengths of 570nm and 600nm after 5 h and 24 h using a Synergy 2 microplate reader (Biotek, Germany).

4.3.4 Data processing and statistical analysis

The growth of the *L. pneumophila* strains in batch cultures was analysed in two different steps. First, raw optical density measurements directly obtained from Bioscreen C were examined as previously described (15). Briefly, blank OD values (only BYE medium) were substracted to all measurements. To normalize the data, each OD measurement was divided by the initial OD of the inoculum (N_t/N_0) and then the natural logarithm of the ratio N_t/N_0 was calculated for each well. In graphs and growth curves representations, the natural logarithm of the ratio N_t/N_0 was shortened as "LnOD600nm". Averages of the five replicates were calculated per strain. Second, after pre-treating the OD measurements as explained, data were analysed using the statistical package "opm" for R (16), using a spline-fit algorithm for curve fitting. Growth kinetics were summarized in three parameters: lag time (λ), growth rate (µ) and maximum cell density (A). These parameters were calculated for each strain at each temperature. Statistical analyses were performed using GraphPad Prism version 5.00

(GraphPad Software, San Diego California USA). Data were checked for normality using D'Agostino and Pearson omnibus normality test. To determine if temperature had significant effects on lag time, growth rate and maximal density non-parametric Kruskal-Walis test and Dunn´s Multiple Comparison test were applied. Student´s t-tests or non-parametric Mann-Whitney tests were applied to compare each growth parameter between environmental and clinical isolates, and between genotypes at each studied temperature. Growth curves were built using GraphPad Prism version 5.00. Multivariate analyses were carried out using Primer version 7.0.9. Growth rates and maximum density values of each strain at each temperature were first normalized by subtracting the median and dividing by the interquartile range of the values of each temperature. Principal coordinate analysis (PCO) was performed from a resemblance matrix that was created by Euclidean distance. One-way Analysis of Similarities (ANOSIM) test was used to infer differences between the groups represented in the PCOs using Primer version 7.0.9.

Intracellular multiplication of *L. pneumophila* strains in THP-1 human macrophages was presented as relative growth. Data were normalized by dividing the CFU/ml obtained at 24 h and 48 h by the number of CFU/ml at 2 h. Uptake levels were calculated as previously defined (17) using the formula [% uptake = (CFU of gentamicin-resistant bacteria/CFU of inoculum) x 100]. To correct for variation between experiments, the uptake was reported as relative to the reference strain *L. pn.* Philadelphia-1, i.e., [relative entry = (% uptake of test strain/ % uptake of *L. pn*. Philadelphia-1) x 100)]. Cytotoxicity of *L. pneumophila* strains to the monolayers of macrophages was calculated as the ratio of the absorbance of the reduced Alamar blue of infected monolayers to that of uninfected ones (18). Specifically, the percentage of cytotoxicity was calculated by the formula [((O2xA1) – (O1xA2) / (O2xP1) – (O1xP2)) x100], where O1 and O2 were the molar extinction coefficients of oxidised Alamar blue at 570 and 600nm, respectively; A1 and A2 were the absorbances of infected cells at 570 and 600nm, respectively; and P1 and P2 were the absorbances of non-infected cells at 570 and 600nm, respectively. Cytotoxicity of each *L. pneumophila* strain was measured in triplicate for each MOI. A *dotA* negative mutant of the strain *L. pneumophila* Philadelphia-1 was used as negative control in the three different infection assays. Kruskal-Wallis non-parametric test was used to test differences between genotypes or clonal complexes during the uptake and at each time point (2h, 24h or 48h) of their intracelular growth. Non-parametric Mann-Whitney and Kruskal-Wallis tests were used to compare the percentage of cytotoxicity between environmental and clinical isolates as well as to compare the cytotoxicity of different serogroups, genotypes or clonal complexes. To assess the differences in cytotoxicity between serogroup 1 and non-serogroup isolates and, in addition, between Gt4(17) clinical and environmental isolates, two tailed unpaired T test was used. Calculations and graphs were generated using GraphPad Prism version 5.00.

4.4 Results

4.4.1 Growth of *L. pneumophila* strains at various temperatures

A set of 63 *L. pneuomphila* isolates, which were previously characterized by their serogroup, MLVA genotype and their virulence potential to human macrophages (**Table S1**), were selected to study their growth in BYE liquid medium at a range of temperatures that varied from 15°C to 45°C. Specifically, 37 isolates were obtained from freshwater systems and 26 had clinical origin. Quantitative growth of environmental and clinical isolates was studied by the analysis of three important parameters that describe bacterial growth in batch culture: growth lag (λ), growth rate (μ) and maximum biomass or maximum cell density (A). *L. pneumophila* presented the characteristic growth response to temperature of mesophilic microorganisms (**Figure 1**).

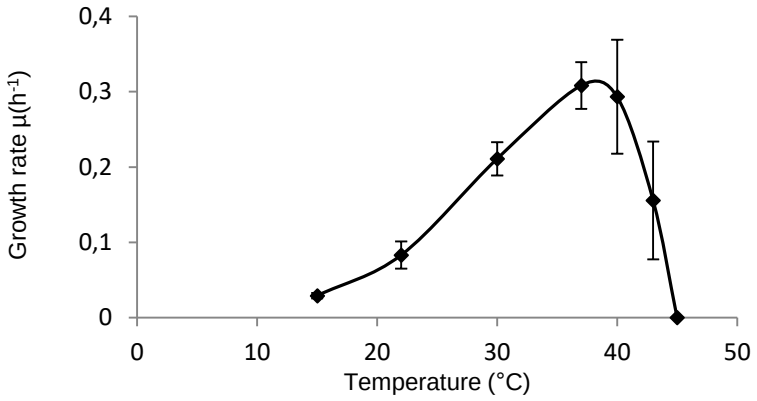
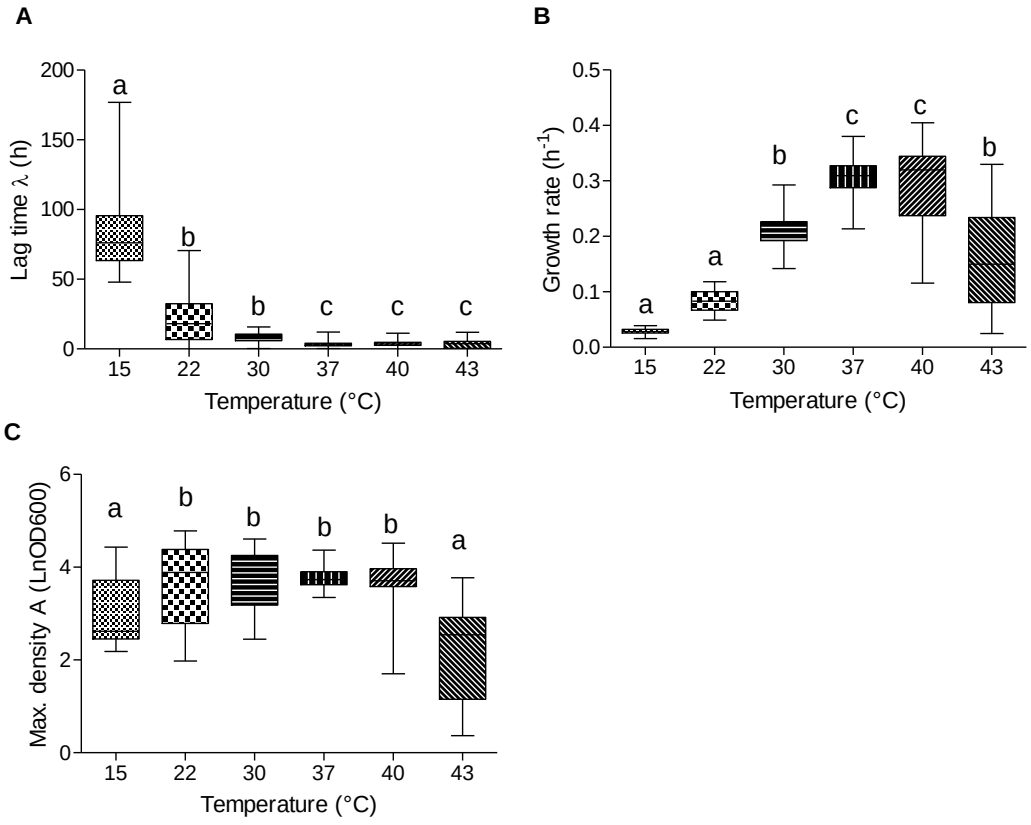


Figure 1. Effect of temperature on the growth rates (μ) of *L. pneumophila* *(*averages of the growth rates of all 63 isolates at each temperature are presented).

The 31 isolates studied at 15°C were all able to multiply at this temperature. Likewise, all 63 isolates studied at the rest of temperatures multiplied between 22°C and 40°C. The upper limit of growth temperature could be stablished in this study at 43°C since at this point only 41 out of 63 isolates multiplied. At 45°C none of the *L. pneumophila* isolates multiplied. At this temperature, the optical density, as measure of the cell density or biomass, did not increase in comparison to the initial inoculum. Instead, it decreased rapidly, signal of denaturation and breakdown of cell components. The study of lag times revealed significant differences of this parameter according to the growth temperature ($P<0.001$) (**Figure 2A**). At low temperatures *L. pneumophila* isolates needed longer times to start growing exponentially. Specifically, the longest lag times were reached at 15°C and 22°C, with averages of 81.9 h and 21.2 h, respectively. On the contrary, the shortest lag time were reached by the 41 isolates that were able to multiply at 43°C, with an average of 2.9 h. As can be seen on **Figure 1**, the optimum growth temperature, at which growth was significantly most rapid, was 37°C. The growth rates varied significantly along the range of temperatures ($P<0.001$) (**Figure 2B**). Below and above 37°C growth rates decreased gradually. As well as the other two parameters, cell densities

reached in the batch cultures varied significantly with the temperature (**Figure 2C**). Cell densities were very similar at 15°C and 43°C, presenting the lowest values. On the contrary, higher cell densities were observed at 22°C, 30°C, 37° and 40°C.

Figure 2. **A**) Lag time (λ), **B**) growth rate (μ) and **C)** maximal cell density (A) of *L. pneumophila* isolates at different temperatures. The growth of 31 isolates was examined at 15°C. 63 isolates were studied at the rest of temperatures. Different letters over the box plots indicate significant differences between temperatures calculated by Kruskal-Wallis non-parametric test and Dunn´s Multiple Comparison test with a confidence level of 95% (Same letters over the box plots of specific temperatures indicate no significant differences between those temperatures for the specific parameter).



4.4.2 Effect of temperature on the growth of environmental and clinical isolates of *L. pneumophila*

Differences could be observed between environmental and clinical isolates by inspecting the growth curves (graphical data) obtained from the batch cultures at the different temperatures (**Figure 3**). In general, environmental strains multiplied more rapidly and efficiently at low temperatures (15°C and 22°C) than clinical isolates. However, at higher temperatures (30°C, 40°C and 43°C) this pattern was reverted and clinical isolates showed higher growth capacities than environmental isolates. At 37°C, the optimum growth temperature, the growth of both groups of isolates became identical. At lower temperatures

clinical isolates exhibited significant longer lag times than environmental isolates (**Figure 4A)**. As the temperature increased, lag periods became even, showing no differences between the groups. On the contrary, clinical isolates presented significantly higher growth rates than environmental isolates at higher temperatures (30°C and 43°C) and significantly lower at 22°C (**Figure 4B**). At 37°C and 40°C, the growth rates of both types of isolates were almost identical, slightly higher in the clinical strains. As it occurred with these two parameters, clear differences were observed between the cell densities of the groups in relation to the temperature (**Figure 4C).**

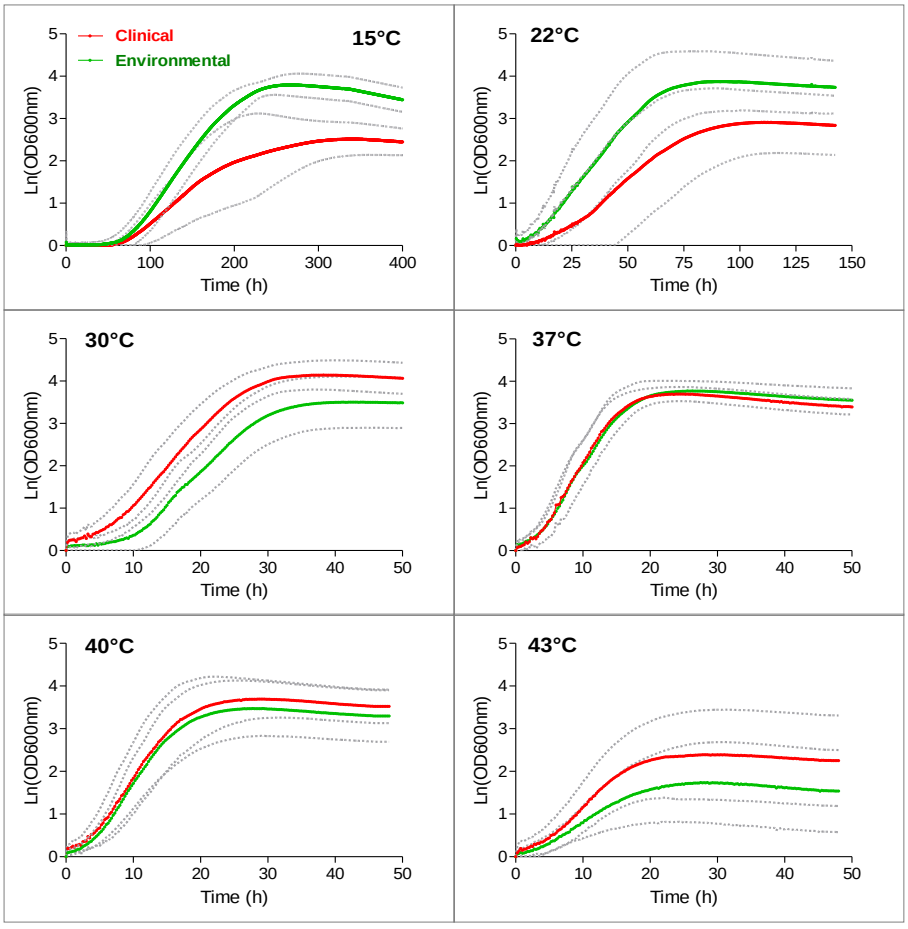
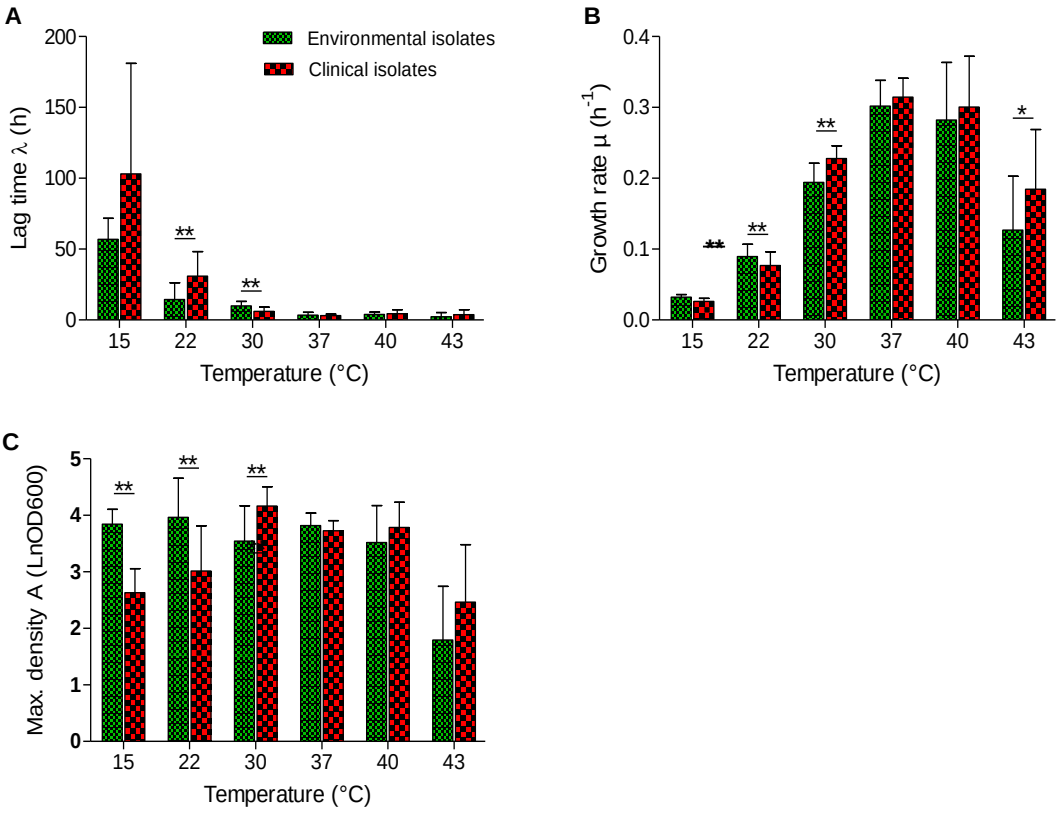


Figure 3. Growth curves representing the average and standard deviations (grey lines) of 37 environmental and 26 clinical *L. pneumophila* isolates at six different temperatures (only 12 environmental and 22 clinical isolates were tested at 15°C). The parameters lag phase (h), growth phase μ (h^{-1}) and maximum density were calculated from the curves and used to infer differences between clinical and environmental isolates.

At 15°C and 22°C, environmental isolates increased significantly in cell densities in comparison to the clinical isolates. However, a shift occurred when the temperature was increased to 30°C. Although at this temperature the differences between the two groups were not as pronounced as at colder conditions, cell densities of clinical isolates became significantly higher than those of the environmental isolates. As temperature increased to 37°C and 40°C both groups reached very similar cell densities. At 43°C a general decrease of the growth of both types of isolates was noticed. Only 54% of environmental isolates were able to growth at this temperature in comparison to the 80.7% of the clinical isolates. Additionally, clinical isolates reached cell

densities significantly higher than those of the environmental isolates. **Figure 5** represents the lag time, growth rate and maximal cell density of environmental and clinical isolates as functions of the temperature. The shift caused by the temperature in the cell densities of both groups can be clearly identified.

Figure 4 A) Lag time (λ), **B**. growth rates (μ) and **C**. maximal cell density (A) of *L. pneumophila* clinical and environmental isolates at different temperatures. The growth of n=31 isolates was examined at 15 °C. n=63 isolates were studied at the rest of temperatures. Asterisks denote significant differences calculated by Kruskal-Wallis non-parametric test and Dunn´s Multiple Comparison test with a confidence level of 95%.



In order to visualize the correlations between the different growth parameters of environmental and clinical isolates and the temperature, multivariate analyses were carried out. No correlation was observed in the principal coordinate analysis (PCO) when the analysis was performed using the three parameters (lag time, growth rate and maximal cell density) (**Figure S1**). However, multivariate analysis using ANOSIM revealed significant differences in the growth rates and maximal densities between clinical and environmental isolates of *L. pneumophila* (R=0.206, P<0.001) (**Figure 6)**. Environmental isolates divided significantly more rapid and reached higher densities than clinical isolates at low temperatures, especially at 15°C. The opposite occurred at high temperatures that favored the multiplication of clinical isolates. At their optimum growth temperature, particularly at 37°C, the maximum densities of clinical and

environmental isolate were highly comparable and both groups could not be distinguished. Interesting, the evenness of the maximal density at 37ºC contrasted with the variation of the growth rates at this temperature, which varied up to two fold (**Figure 7**). A good correlation was observed between growth rate and maximal density for the rest of temperatures (**Figure 7**). Multivariate analysis applying only the growth rates at all different temperatures showed the same distinction between clinical and environmental isolates (**Figure S1**). However, multivariate analyses did not show clear differences between the two types of isolates along the range of temperatures when comparing exclusively their maximal densities (**Figure S2**).

Figure 5. Overview of temperature and growth response of environmental and clinical isolates of *L. pneumophila***.** Averages of **A)** lag time, **B**) growth rates and **C)** maximal cell densities of environmental and clinical isolates as functions of the temperature.

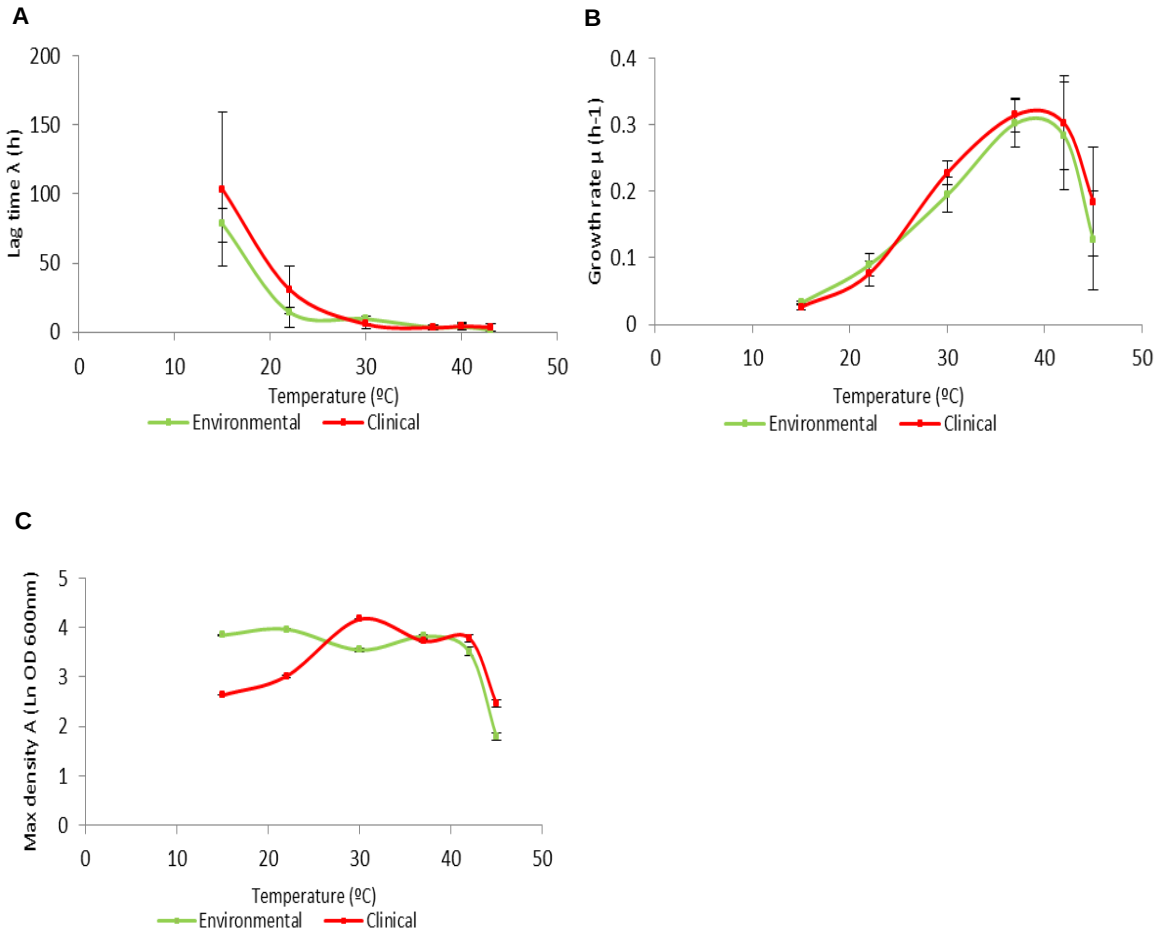


Figure 6. Principal coordinate analysis (PCO) of the growth rate (μ) and maximal density (A) of 37 environmental and 26 clinical *L. pneumophila* isolates growing at six different temperatures. Significant differences between environmental and clinical isolates were detected (ANOSIM R=0.206, P<0.001)

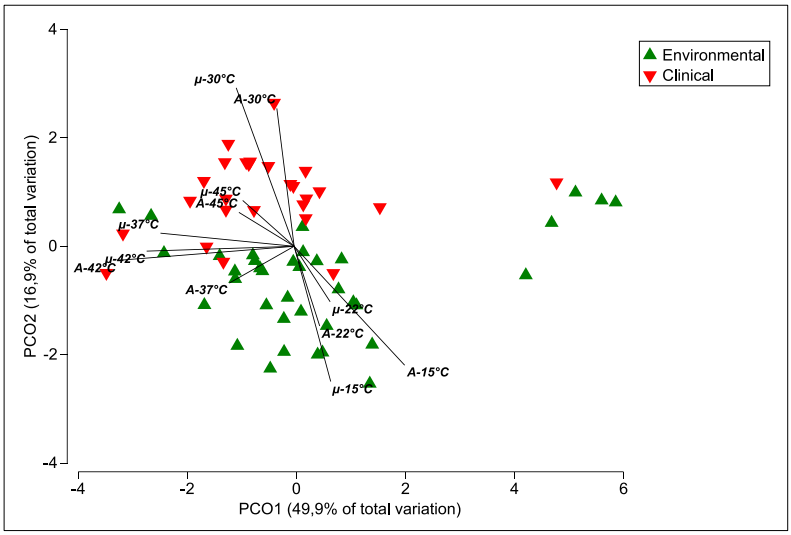
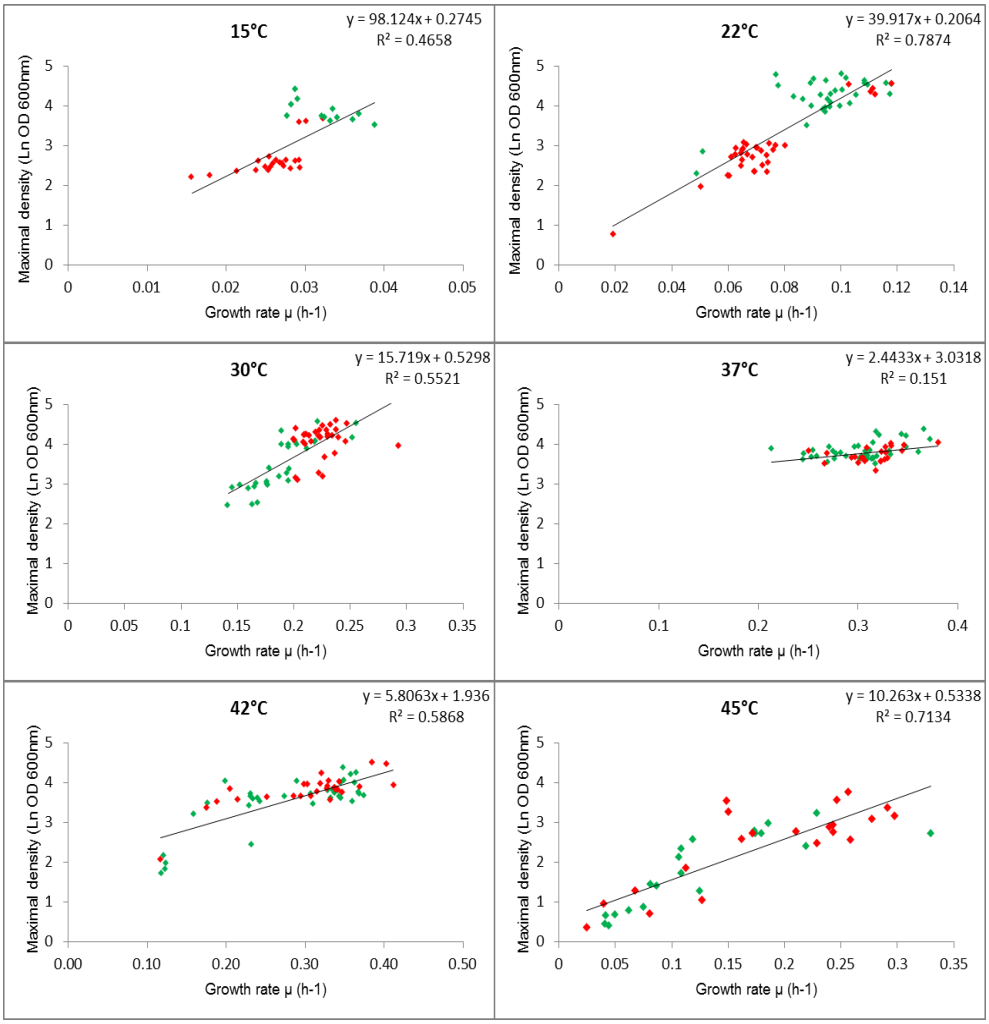


Figure 7. Correlation between cell density and growth rate at different temperatures.



4.4.3 Effect of temperature on the growth of different *L. pneumophila* genotypes

The 25.4% (n=16) of the *L. pneumophila* isolates used in this study belonged to MLVA-8(12) genotype Gt4(17), which has been shown to correspond to the world wide distributed ST1. The two main growth parameters that define growth, growth rate and cell density, were used to compare the growth kinetics of Gt4(17) to the rest of genotypes (here named as non-Gt4(17) (**Figure 8**). Comparisons to other specific genotypes were statistically not possible due to the high diversity of the non-Gt4(17) group, which accounted with a low number of isolates of each genotype. No significant differences were observed between the lag times of Gt4(17) and other genotypes at low or high temperatures when compared by t-test or its non-parametric variant (**Figure 9A**) The same phenomenon was observed in terms of growth rate and cell density (**Figure 9B and 9C**). However, the analysis of similarity revealed significant differences between the growth rates and maximum density of Gt4(17) and non-Gt4(17) (ANOSIM R=0.373, P<0.001) when these parameters were used to compare both groups at all temperatures tested (15°C, 22°C, 30°C, 37°C, 40°C and 43°C). 43.6% of the total variance between the isolates was explained by the first component (PCO1) represented in the x axis of the PCO plot (**Figure 10A**). According to this component, low temperatures (15°C and 22°C) would favor the growth of Gt4(17) isolates.

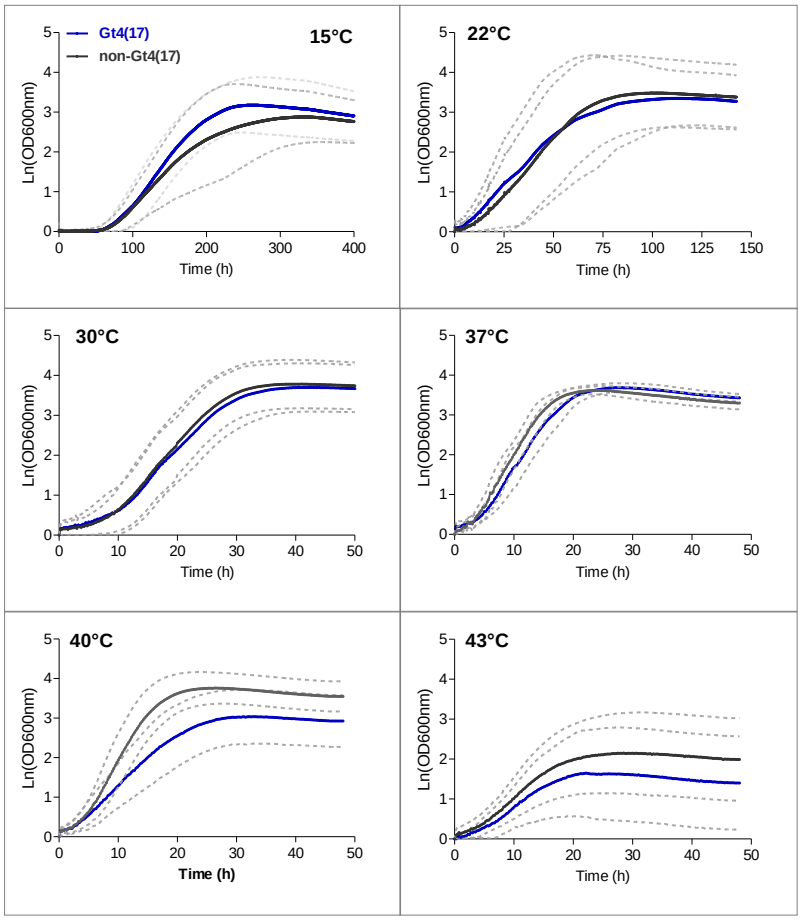


Figure 8. Growth curves representing the average and standard deviations of 16 Gt4(17) isolates and 47 non-Gt4(17) *L. pneumophila* isolates at 22°C, 30°C and 43°C. The average and standard deviations of 10 Gt4(17) and 24 non-Gt4(17) were compared at 15°C. Parameters growth rate and maximum density were calculated from the curves and used to infer differences between clinical and environmental isolates

Figure 9. **A**) Lag time, **B**) growth rate and **C**) maximum cell density of Gt4(17) compared to other MLVA genotypes (non-Gt4(17) at 15°C, 22°C, 30°C and 43°C. The group of non-Gt4(17) genotypes was composed of 47 isolates that belonged to 14 MLVA-8 genotypes. At 15°C only 10 Gt4(17) and 24 non-Gt4(17) isolates were studied. To compare Gt4(17) and non-Gt4(17) isolates at each temperature, t-test or Mann-Whitney test were used according to normality of the data.

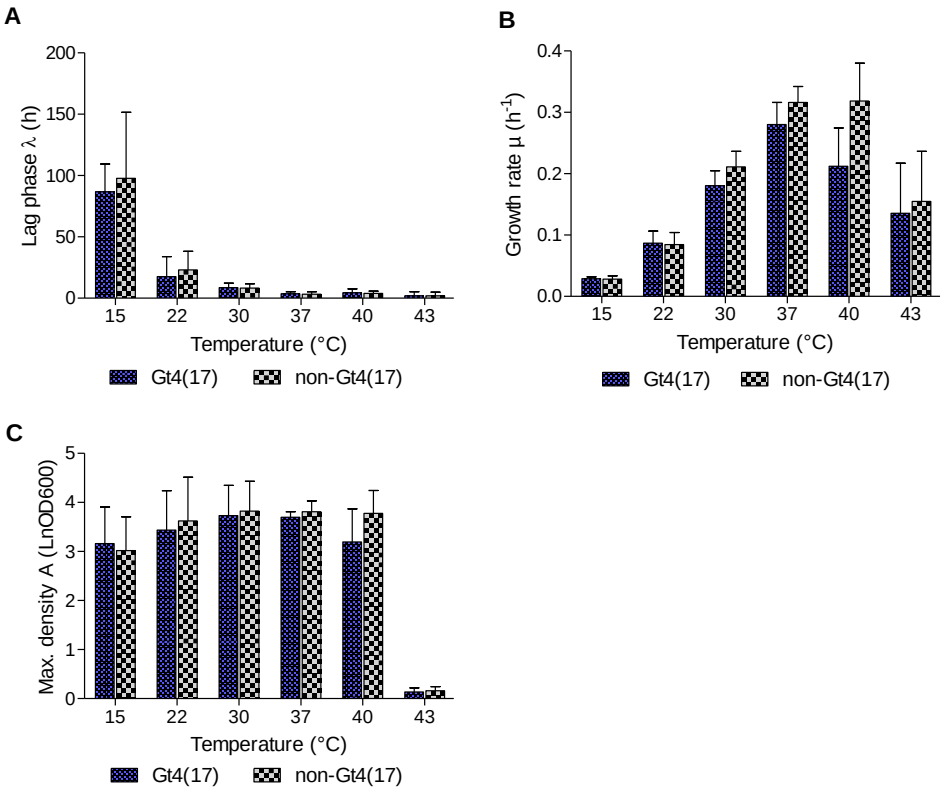
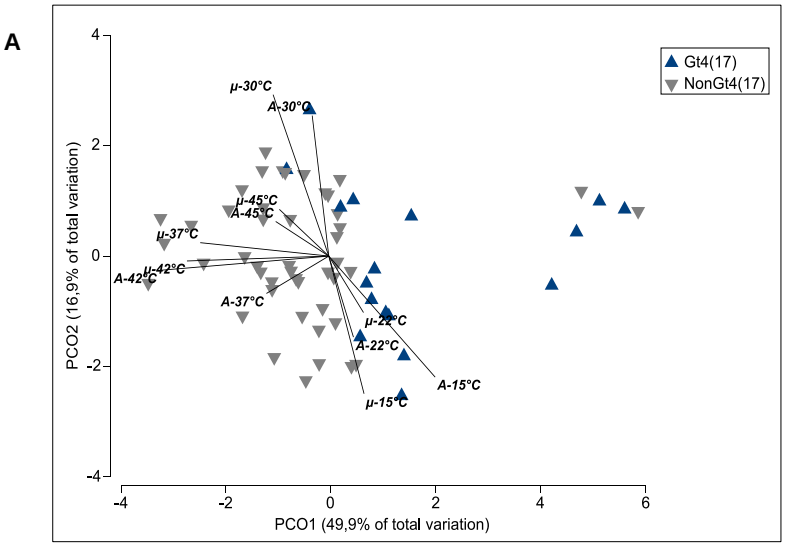
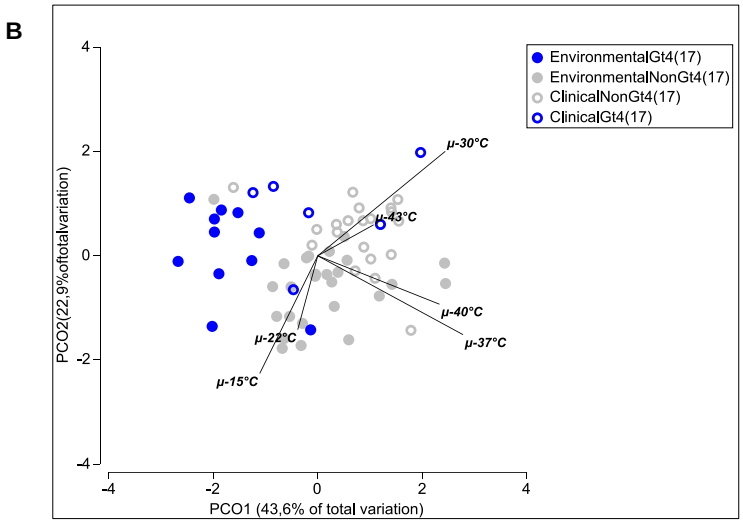


Figure 10. A) Principal coordinate analysis (PCO) of the growth rate and maximum density of 63 *L. pneumophila* isolates classified according to their MLVA genotype. Gt4(17) isolates were compared to the rest of the genotypes, which were grouped as non-Gt4(17). The analysis of similarity revealed significant differences between Gt4(17) and all the other genotypes (ANOSIM R=0.373, P<0.001).



B) Principal coordinate analysis (PCO) considering exclusively the growth rate of 63 *L. pneumophila* isolates classified according to their MLVA genotype. Analysis of the variance showed greater differences between Gt4(17) (blue filled and unfilled dots) and the rest of genotypes (grey filled and unfilled dots) when only this parameter is analyzed (R=0.503, p<0.001). Significant differences were also observed between environmental (filled blue dots) and clinical (unfilled blue dots) Gt4(17) isolates (R=0.297, p<0.05). The rest of non-Gt4(17) isolates were as well differentiated in environmental and clinical isolates (grey filled and unfilled dots, respectively).



Strains of genotype Gt4(17) have been found frequently in the environment but has also been responsible of multiple cases of legionellosis (5). To determine possible growth differences between clinical and environmental isolates of this genotype, growth rates and maximum cell densities were compared at six different temperatures. **Figure 11** shows the growth curves of the two types of strains of Gt4(17). The lag time of clinical and environmental isolates differed significantly at 22ºC and 30ºC (**Figure 12A**). Clinical Gt4(17) isolates needed longer periods of time than Gt4(17) environmental isolates to start growing exponentially at 22ºC (P<0.001). The opposite occurred with the increase of temperature to 30ºC, lag times of clinical isolates were significantly shorter in comparison to those of environmental isolates (P<0.05). Significant differences were observed in the growth rates of environmental and clinical Gt4(17) isolates exclusively at 30°C (**Figure 12B**). Gt4(17) clinical isolates could multiply more rapidly than environmental isolates at this temperature. Analysis of similarity also showed significant differences between the growth rates of clinical and environmental Gt4(17) isolates when only this parameter was represented by PCO (ANOSIM R=0.297, p<0.05) (**Figure 10B**). When the growth densities were considered, it was observed that environmental Gt4(17) isolates reached significantly higher cell density at low temperature (15°C) than clinical isolates (**Figure 12C**). In contrast, Gt4(17) clinical isolates reached significantly higher (P<0.05) cell density than environmental isolates at 30ºC (**Figure 12C**). At 43°C only 20% (two out of ten) of the environmental Gt4(17) isolates were able to grow in contrast to the 66.6% (four out of six) clinical isolates. In **Figure 11** it can be seen how environmental isolates were able to form

higher amounts of biomass at low temperatures while clinical isolates grew more efficiently at higher temperature.

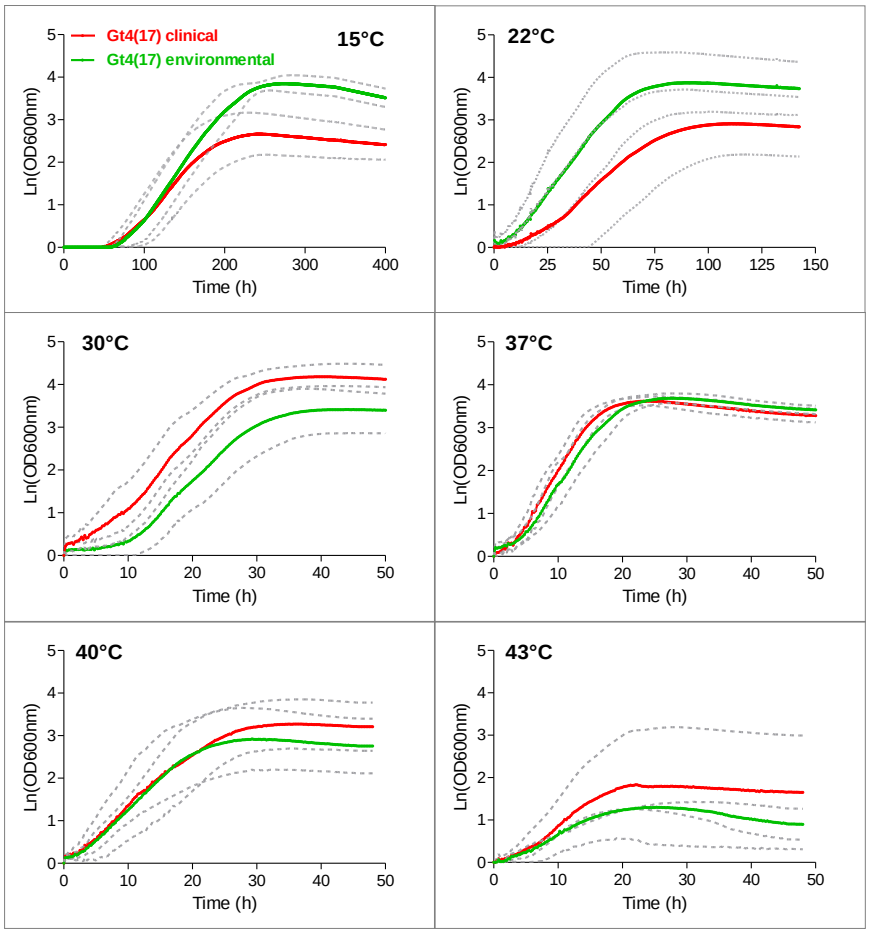
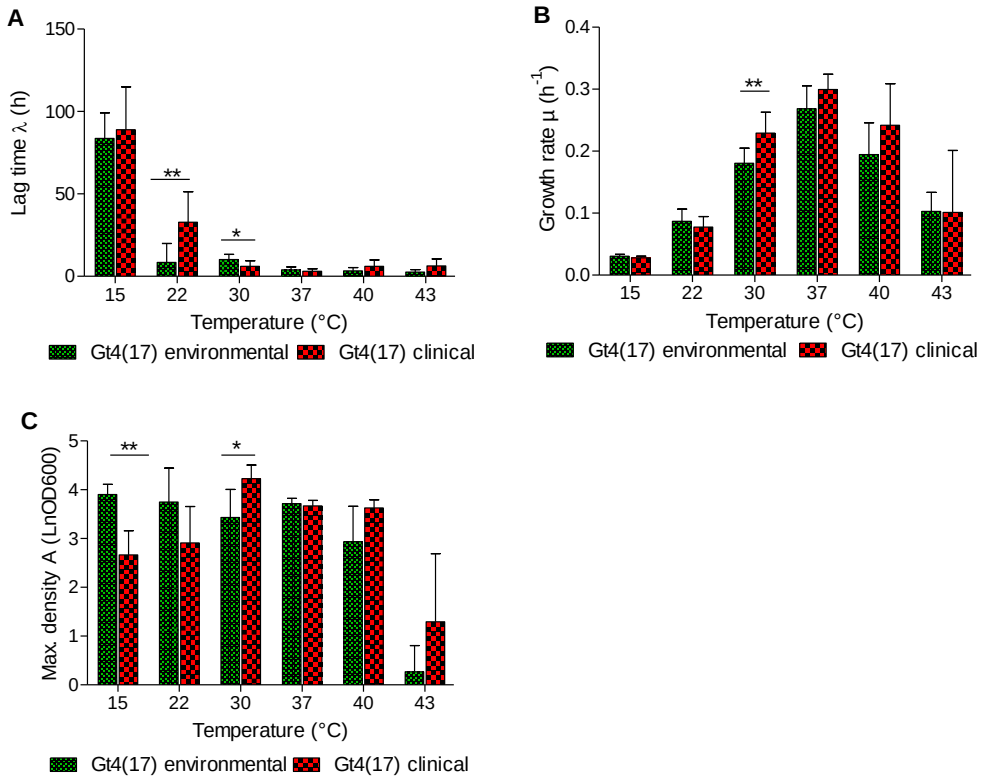


Figure 11. Growth curves representing the average and standard deviations of 6 environmental and 10 clinical Gt4(17) isolates at 15°C, 22°C, 30°C and 43°C. The average and standard deviations of 4 environmental and 6 clinical Gt4(17) were compared at 15°C. The parameters growth phase μ (h^{-1}) and maximum density were calculated from the curves and used to infer differences among clinical and environmental isolates.

Besides comparing the growth of environmental and clinical *L. pneumophila* isolates and the growth of specific genotypes, the differences in the growth between serogroups was compared. In particular, multivariate analyses were carried out in order to infer differences between serogroup 1 isolates and the rest of serogroups contained in the dataset (**Figure S3**). No differences were observed in the growth of isolates of distinct serogroup. In addition, it was also confirmed that environmental strains isolated in Europe had a similar growth behavior to the environmental strains isolated in the Middle East (**Figure S4**). The group composed of environmental strains was homogeneous, independently of the geographical origin of the strains.

Figure 12. Comparison of growth rates (A) and maximum cell density (B) of environmental (n=6) and clinical (n=10) Gt4(17) isolates at 15°C, 22°C, 30°C and 43°C. At 15°C only 4 environmental and 6 clinical Gt4(17) isolates were studied. Asterisks indicate significant differences according to Mann-Whitney test (P<0.001).



4.4.4 Intracellular growth and uptake of *L. pneumophila* strains by THP-1 macrophage-like cells

An infection model that allowed the study of the uptake, intracellular growth and cytotoxicity in the human macrophage cell line THP-1 was stablished to investigate the intracellular behavior of genetically and phenotypically distinct *L. pneumophila* strains. Uptake, intracellular growth and cytotoxic assays were initially assessed in a group of 21 environmental *L. pneumophila* strains with different phenotypic and genetic characteristics (serogroups and MLVA genotypes). The reference strain *L. pneumophila* Philadelphia-1 ATCC33152 and its Icm/Dot deficient *dotA* mutant were used as positive and negative controls, respectively. Specifically, the 21 environmental strains were grouped according to their serogroup, MLVA genotype and to their MLVA Clonal Complex (VACC) in order to assess the inference of these genetic features in the intracellular growth of the strains. To compare the cytotoxicity, uptake and intracellular growth of the strains grouped in different clonal complexes, only those VACCs composed of at least three different genotypes and two different serogroups were selected. As shown in **Figure 13A**, no significant differences were observed in the cytotoxicity between strains of different serogroups and genotypes after 5 h of infection. Cytotoxicity levels were

highly consistent at the four different MOI applied. Uptake levels were highly variable between the genotypes, however, only those of Gt12(84), which belonged to serogroup 8 (sgp8), were significant in comparison to the *dotA* mutant ($P<0.05$) (**Figure 13B**). These results suggested that there could be potential differences in the efficiency of different genotypes to enter the macrophages.

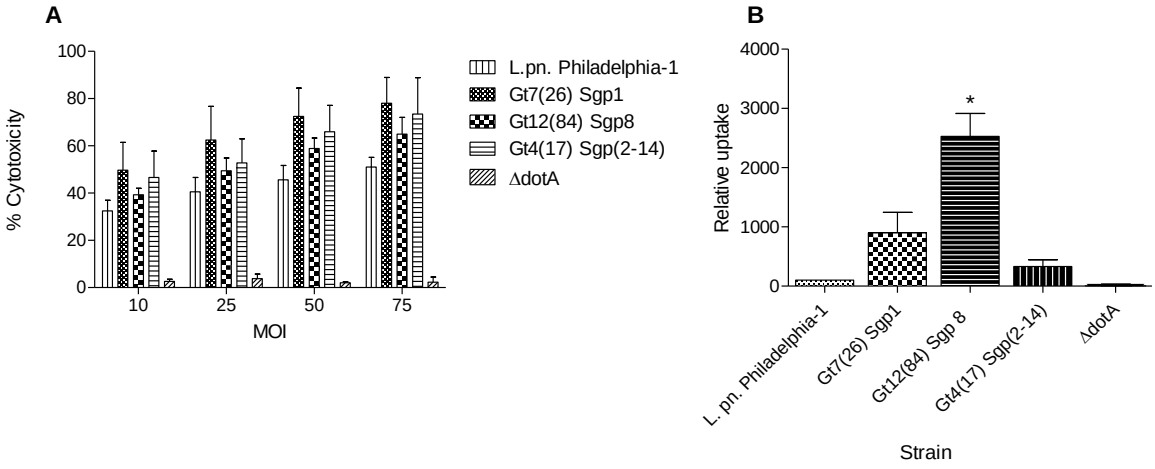


Figure 13. Cytotoxicity and relative uptake of 21 environmental *L. pneumophila* isolates grouped by their MLVA-8(12) genotype and and serogroup. **A)** Cytotoxicity to THP-1 macrophages of three genotypes with their corresponding serogroups, the reference strain *L. pn*. Philadelphia-1 and its *dotA* mutant as negative control at four distinct MOI (10, 25, 50 and 75) after 5 h post-infection. **B)** Relative uptake by THP-1 macrophages by three distinct genotypes in comparison to the reference strain *L.pn*. Philadelphia-1. The uptake of *L.pn*. Philadelphia-1 was arbitrarily set to 100. Data points and error bars represent the mean and standard deviations, respectively, of triplicates of three isolates of each genotype.

As shown in **Figure 14**, no differences were observed in the intracellular replication between strains of different serogroups and genotypes (**A**) or among strains that belonged distinct clonal complexes (**B)** after 1 h, 24 h or 48 h post infection. Only significant differences were observed among the strains and the *dotA* mutant at 24 h and 48 h. The clinical reference strain showed a highly similar pattern of intracellular multiplication to the environmental strains. Despite the great potential of the intracellular growth and uptake assays, cytotoxicity assay was preferred for the analysis of the large dataset of environmental and clinical *L. pneumophila* isolates utilized in this study as it enabled a rapid overview about their infectivity.

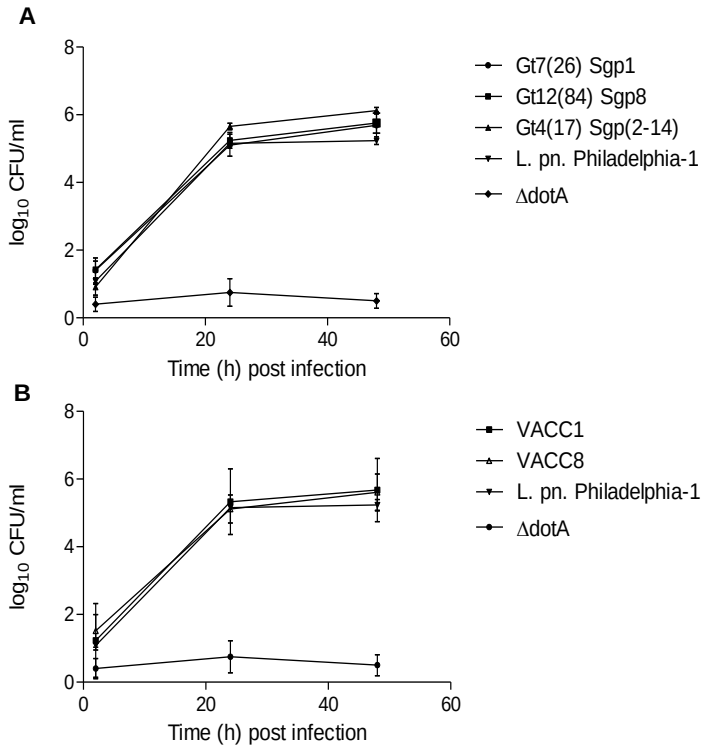


Figure 14. THP-1 macrophage infection at MOI 1 by *L. pneumophila* strains grouped according to their MLVA genotype (**A**) and their clonal complex (**B**). In A, at least three distinct *L. pneumophila* strains of the same serogroup and genotype were compared. In B, VACC8 was composed of eight *L. pneumohila* strains of three different serogroups and four genotypes. VACC1 was formed by seven strains of two serogroups and three genotypes. Each data point represents the mean and standard deviation (error bars). Infections were performed in triplicates for each strain.

4.4.5 Cytotoxicity of *L. pneumophila* strains to THP-1 macrophage-like cells.

Cytotoxicity of a dataset composed of 85 *L. pneumophila* environmental and clinical isolates was examined by the infection of THP-1 macrophages during 1 h at four different MOI (10, 25, 50 and 75) and taking absorbance measurements at 5 h and 24 h of incubation with Alamar blue after infection. In general, cytotoxicity decreased after 24 h in comparison with the cytotoxicity observed after only 5 h post infection. (**Figure S5**). The different MOIs presented proportional results. The percentage of cytotoxicity increased proportionally in all isolates with the use of higher MOI. However, significant differences among strains were only able to be detected when low MOI, as 10 and 25, were applied. Thus, to perform comparisons among strains and avoid data redundancy only data from measurements at 5h using MOI 10 were considered for further analysis.

The group of 85 *L. pneumophila* strains contained 51 environmental and 33 clinical isolates of different serogroups and genotypes (in addition to the *L. pneumohila* Philadelphia-1 dotA mutant strain) (**Table S1**). Environmental isolates produced significantly higher cytotoxicity to THP-1 macrophages than clinical isolates. Nevertheless, the range of cytotoxicity differed considerably among isolates within each group, oscillating between 20% and more than 60% (**Figure 15**).

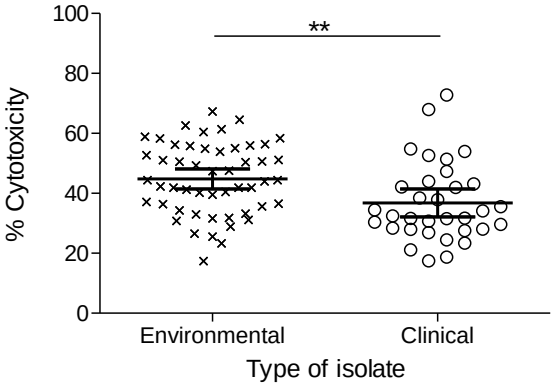
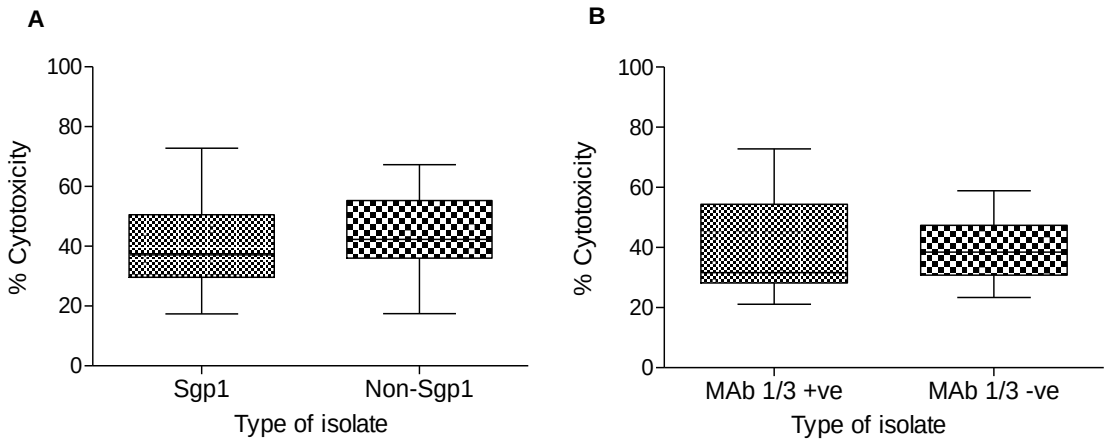


Figure 15. Cytotoxicity of 51 environmental and 33 clinical *L. pneumophila* isolates at 5 h postinfection and incubation with Alamar blue (MOI 10). Cytotoxicity of each isolate was examined in triplicate. Asterisks denote statistically significant differences (P<0.05) by Mann-Whitney U test.

According to epidemiological studies (19) serogroup 1 is the most virulent of the more than 15 serogroups that have been described for the species *L. pneumophila* since it is the most frequent cause of legionellosis. Infections performed comparing different serogroups in human macrophages as well as in the natural hosts also suggested the higher virulence potential of serogroup 1 over other serogroups (20). Consequently, the differences in cytotoxicity among environmental and clinical isolates could be due to the presence of certain serogroups. However, 93% (n=31) of the clinical isolates used in this study, represented in **Figure 15,** were characterized as sgp1 in contrast with the 47% (n=24) of the environmental isolates. The cytotoxicity to THP-1 macrophages was highly similar between sgp1 and non-sgp1 isolates (**Figure 16A**) and no significant differences were found between both groups. Population studies have also revealed that not only the sgp1 strains possessed higher virulence that non-sgp1strains (5) but the virulence of the sgp1 strains can be as well influenced by modifications in the lipopolysaccharide (LPS) epitope (21). The LPS patterns are detected by monoclonal antibodies leading to the classification of sgp1 strains into distinct monoclonal subgroups. Monoclonal subgroups are classified into MAb 3/1 negative and MAb 3/1 positive according to their virulence, being MAb 3/1 positive strains commonly isolated from patients but rarely found in freshwater systems (5). The 29 *L. pneumohila* sgp1 isolates from this study which were subtyped by monoclonal subgrouping were clustered by their MAb 3/1 classification to analyze their cytotoxicity to THP-1 human macrophages. Results showed that MAb 3/1 positive and negative strains have highly comparable levels of cytotoxicity (**Figure 16B**). No significant differences were observed between both groups.

Figure 16. Analysis of the effect of the serogroup and the monoclonal subgroup to the cytotoxicity caused to THP-1 macrophages. **A**) Comparison of 55 sgp1 and 29 non-sgp1 *L. pneumophila* isolates. Two tailed unpaired T test showed no significant differences among the groups (P>0.05). **B**) Comparison of sgp1 isolates with different monoclonal antibody (MAb) subtypes. Two tailed unpaired T test revealed again no significant differences among the two groups (P>0.05).



A more detailed comparison was carried out in order to search for general features, as specific serogroups or genotypes that could influence the virulence of the strains during the infection to human macrophages. The cytotoxicity level of different serogroups was compared (**Figure 17**). At least 4 isolates were included in each group. Isolates belonged to different MLVA genotypes, in the cases of sgp1, sgp3 and sgp4. Non-sgp1 isolates whose serogroup was not further identified were grouped together and compared to the rest as sgp 2-14. Overall, significant cytotoxicity differences were found between the serogroups (P<0.05). Specific pairwise comparisons noted significant dissimilarities between sgp3 and sgp4 and between sgp4 and the reference strains. Sgp1 and sgp 2-14, the richest groups in terms of number of strains and genotypes, showed a higher variability in cytotoxicity.

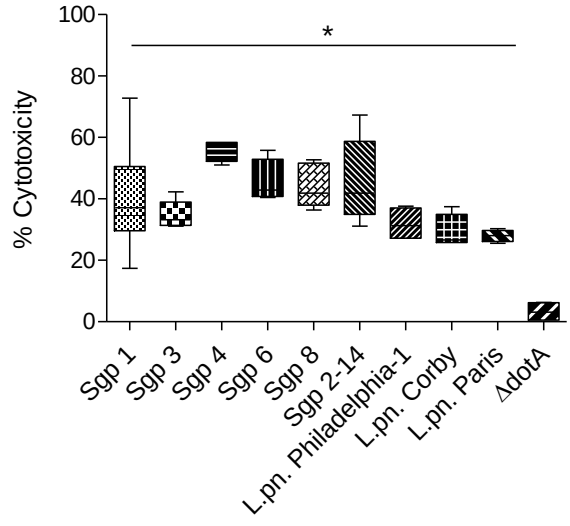
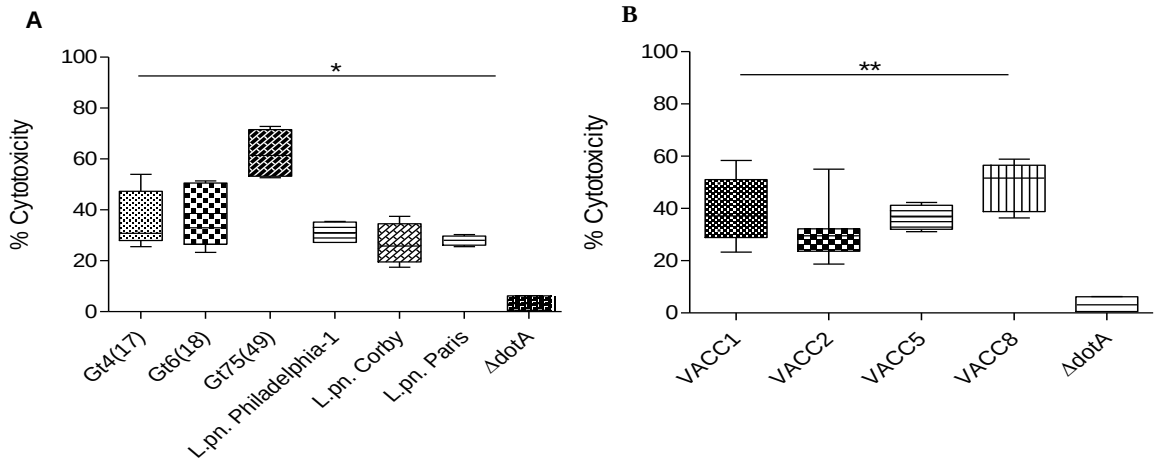


Figure 17. Cytotoxicity to THP-1 human macrophages of different serogroups and three sgp1 reference strains at 5h post infection (MOI 10). Significant differences were found between the serogroups (P<0.05) by the non-parametric Kruskal-Wallis test. Mutant strain *dotA* functions as negative control.

Figure 18. A) Evaluation of cytotoxicity to THP-1 macrophages of Sgp1 strains classified in different MLVA genotypes. Kruskal Wallis significance test showed substantial differences between the diverse genotypes and reference strains (P<0.05). **B**) The different clonal complexes or clusters of genotypes (VACC) showed as well noticeable differences between them regarding the virulence to THP-1 human macrophages (Kruskal Wallis significance test, P<0.05).



Since sgp1 is the most isolated serogroup among clinical strains but it is also very persistent in the freshwater systems, multiple sgp1 strains of different MLVA genotypes were compared in order to discern differences in the virulence potential of distinct genotypes, independently of the serogroup (**Figure 18A**). A minimum of four different isolates of Gt75(49) and seven of Gt4(17) and Gt6(18) were analyzed. Gt75(49), which was represented by four clinical isolates, exhibited higher cytotoxicity than the reference strains and the environmental genotypes Gt4(17) and Gt6(18). These results showed the high variability in infectivity potential of the strains with the same serogroup. To estimate specifically the virulence of the isolates that belong to the different clonal complexes, only those clonal complexes including different serogroups and genotypes were selected. Significant variances were observed among the clonal complexes (P<0.05), especially among VACC2 and VACC8, which were composed of five and three distinct MLVA-8(12) genotypes respectively (**Figure 18B**).

The cytototoxicity to THP-1 human macrophages of the same genotype isolated from patients or from the environment was evaluated. In particular, the cytotoxicity of environmental and clinical isolates of Gt4(17) genotype, which corresponded to the highly abundant ST1, was compared (**Figure 19**). The virulence did not differ significantly between environmental and clinical Gt4(17) isolates within all MOI tested.

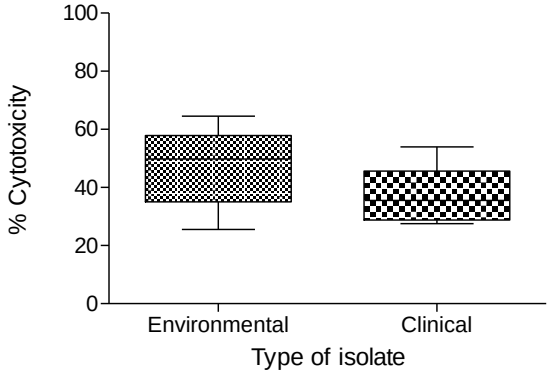


Figure 19. Cytotoxicity to THP-1 human macrophages of 17 environmental and 9 clinical isolates of Gt4(17) after 5 h of incubation with Alamar blue (MOI 10). Each isolate was analyzed in triplicates. Two tail unpaired T-test revealed no significant differences among the groups (P>0.05).

4.5 Discussion

4.5.1 Effect of temperature on growth of environmental and clinical strains of *L. pneumophila*

Temperature is one of the most important environmental factors affecting the metabolism of microorganisms since bacteria cannot regulate their temperature. Temperature mediates changes in growth, development and pathogenesis of the bacteria (22, 23). Water temperature has been considered one of the major impact factors of contamination by *Legionella* (24). Microbial batch cultures constitute a basic microbiological method that allows addressing important physiological questions, such the growth response to controlled environmental factors, e.g. temperature. By cultivating bacteria, essential parameters in their growth cycle, such as lag time, growth rate and maximum density or yield, can be determined. For every microorganism, there is a minimum and maximum temperature below and above which growth is not possible and an optimum temperature at which growth is fastest. In this study it was shown that *L. pneumophila* responded to temperature like a typical mesophilic bacterium. The upper temperature limit was stablished at 43°C. Only 65% (n=41) of the isolates could grow at this temperature and none of them did grow when temperature was increased to 45°C. The lower limit was not finally determined. Although growth was significantly decelerated at 15°C, all isolates tested were able to grow at this temperature. The optimum growth temperature was 37°C. At 37°C the growth was most rapid and the highest cell density was reached. Below and above this temperature, cell density and growth rate decreased. Konishi et al. 2006 (25) already reported that cell growth in liquid culture decreased markedly at temperatures above 44°C.

Examination of the growth kinetics of environmental and clinical *L. pneumophila* isolates revealed that temperature affected the growth behaviour of both groups of strains (**Figure 4**) differently. Generally, environmental strains presented higher growth rates and cell densities, as well as shorter lag times, than clinical strains at low temperatures (15°C and 22°C). With the increase of the temperature to 30°C this pattern shifted and the growth rates and cell

densities reached by clinical strains exceeded those of the environmental ones. Specially, significant differences were observed in cell density between both groups at this temperature. At 43°C, growth capacities of clinical strains were superior in comparison to environmental strains. Moreover, at this limit temperature only 54% of the environmental strains exhibited growth in comparison to the 80.7% of the clinical strains. Interestingly, both groups showed very similar growth kinetics around the optimum growth temperature (37°C and 40°C). The effect of the temperature on growth can be explained in terms of temperature dependence of the structure of cell components, especially proteins and lipids, and temperature dependence of metabolic reaction rates. In this way, growth rate can be affected by changes in the reaction rates, which in turn depend on the activation energies of the reactions. These changes can produce many other additional effects on metabolic regulatory mechanisms, specificity of enzyme reactions, cell permeability and cell composition. For example, it has been demonstrated that the metabolic activity associated with cell division is likely to be affected by temperature (26). Konishi et al. (2006) (25) compared the growth in liquid medium as well as in BCYE plates of three serogroup 1 *L. pneumophila* strains, two of them of clinical origin and the type strain ATCC 33152, at temperatures between 30°C to 47°C. They noted that the clinical strains formed shorter filaments between 39°C and 40°C. Bacterial elongation and filaments formation has been shown to be related to the function of constitutional enzymes for bacterial binary fission (26). These results would indicate that enzymes of the two clinical strains probably kept their functional integrity at higher culture temperature (39°C) than the type strain (37°C).

Selective pressures provided by diverse habitats during their recent evolution may explain this physiological diversity of *L. pneumophila* strains in relation to temperature. Roller & Schmidt (2015) (27) have described how efficient growth can have very important ecological implications. Growth rate, defined as the number of cells produced per time unit, is considered one of the most important components of the fitness in most environments, and therefore an essential trait in the life of microorganisms. Additionally, the efficiency of growth is equally important to the growth rate in the adaptation of strains to the environment and plays a very important role in the survival and persistence of the microorganisms when environmental conditions change and resources become limited. Efficiency of growth has been usually described in terms of yield, that is to say, the number of cells or biomass produced per unit of resource consumed. In this study, the amount of resources provided to the strains, in form of BYE medium, was constant. Therefore, the biomass produced (or cell density, as it has been termed during this chapter) could be directly related to the growth efficiency of the strains. Here, high growth rates were followed of high growth efficiency at all tested temperatures. At 15°C and 22°C the growth rates and growth efficiency of environmental strains were significantly higher than those of the clinical strains. At high temperatures (30°C and 43°C) the opposite occurred and both growth rate and efficiency of clinical strains were higher in

comparison to those of environmental strains. Moreover, independently of the type of strains, good correlations were observed between the growth rate and the growth efficiency (**Figure 7**) at low and high temperatures (not at 37°C where the growth efficiency is maximum). Independently of having higher growth rates or being metabolically more efficient, environmental strains would be favoured at low temperatures over clinical strains and clinical strains would be favoured at high temperatures. The results in this study have also revealed the effect of temperature on the growth at genotype level. Gt4(17), genotype corresponding to ST1, presented significant higher growth rates at low temperatures (15°C and 22°C). The study by Rodríguez-Martínez et al. (2015) (10) supported the findings presenting here. They studied seasonally the presence of *L. pneumophila* MLVA-8 genotypes and the environmental factors affecting a local drinking water distribution system. Gt4 [here Gt4(17)] due to the use of MLVA-8(12) combined genotyping) was dominant in the system, both in water and in biofilm, and prevailed during the seasons. This genotype was predominant at low temperatures, about 20°C, and it did not show any correlation to any of the other environmental parameter analysed, such as pH or chlorine levels. Its presence was primarily triggered by the temperature of the system. Interesting, it was always detected in those water samples with high *Legionella* counts, and most likely those *Legionella* counts were, in fact, Gt4. Schwake et al. (2015) (28) have recently shown that *Legionella* populations in tap water were more stable at 25°C than at 4°C and 32°C. Previously, Ohno & Kato (2003) (29) demonstrated that *L. pneumophila* exhibited longer potential survival without loss of cultivability in microcosms with lower temperatures (25°C). This could suggest that Gt4(17) strains are abundant in the environment due to their general adaption to low temperatures, which can be found, for instance, in the cold water of that supply drinking water distribution system, as remarked by Rodríguez-Martínez et al. (2015) (10). Comparisons of the growth kinetics of environmental and clinical isolates of genotype Gt4(17) confirmed that Gt4(17) clinical isolates were better adapted to high temperatures, and, thus, followed the general trend. This adaptability to higher temperatures might explain the fact that Gt4(17) or ST1, being one of the most abundant sequence types isolates from the environment worldwide, has the capability of producing sporadic cases of legionellosis, especially in elderly immunocompromised patients (30, 31). At clonal level, the high spread around Europe and Middle East of VACC1, whose representative strain is *L. pneumophila* Paris, might be due to the adaptation of its strains to low temperatures. Rodríguez-Martínez et al. (2015) (10) also showed that the other genotype that was found in their water system, Gt15, was always related to the highest temperatures of the system (45.1°C) and low *Legionella* counts were observed where Gt15 was detected. Gt15 belonged to clonal complex VACC5, which has been found to contain clinical isolates from Germany and other European countries.

As explained in Roller & Schmidt (2015) (27), in natural environments other aspects as spatial heterogeneity, availability of resources and temporal resource dynamics could also

influence the selection based on growth. They proposed that these factors influence the competition between individuals and favour those microorganisms that could grow using the resources more efficiently. Yet, in the case of *L. pneumophila,* temperature may influence in turn both resources and its temporal dynamics. Due to the harsh conditions of most natural freshwater environments, most of the time these bacteria live associated with biofilms (32). In the case of *L. pneumophila*, its establishment and persistence in natural and man-made environments is facilitated by biofilm formation and colonization within multispecies microbial communities (33, 34). Previous studies have revealed that *L. pneumophila* was able to replicate extracellularly in the biofilm matrix by using extracellular products released by other bacteria and algae (35). In addition, *L. pneumophila* has the ability to obtain carbon and energy sources from dead organic material, such as other microorganism, possibly produced after disinfection measures (36). Nevertheless, the main route for *L. pneumophila* replication, and thus, for obtaining nutrients and resources, occurs within protozoa. The high concentration of microorganisms within biofilms makes it possible that free-living protozoa can pray and feed on bacteria and *Legionella* can parasitize and multiply within protozoans, contributing to the prokaryotic-eukaryotic co-evolution (37). The efficiency with which these pathogens exploit the available host nutrients for their proliferation is of high relevance and has been called "nutritional virulence". Since the host can complicate the access to the nutrients, pathogens have adapted diverse metabolic routes to successfully use what is available and multiply (38, 39).

Co-culture and in-vitro infection studies have demonstrated the active predation of *Legionella* spp. by protozoa (40). On the other hand, the capability of *L. pneumophila* to parasitize and grow within a wide range of protozoa species has been demonstrated and the dependence of those interactions on physical conditions of the system, such as temperature (41). Buse & Ashbolt (2011) (42) examined the potential effect of in-premise plumbing temperatures (24°C, 32°C, 37°C and 41°C) on the growth potential of different *L. pneumophila* strains within common free-living amoebae. They also examined the effect of the temperature on the growth of amoebas in the presence of prey bacteria. *Acanthamoeba polyphaga* showed growth between 24°C and 32°C, *Hartmannella vermiformis* between 32°C and 37°C and *Naegleria fowleri* from 32°c to 41°C. Below the minimum growth temperature amoebal trophozoites encysted within few days. Above their upper growth limit (32°C, 37°C and 41°C, respectively) encystment was followed rapidly by lysis of the cells. On the other hand, growth of *L. pneumophila* strains was dependent on the host (i.e *L. pneumophila* strain Bloomington-2 replicated successfully in the presence of *A. polyphaga* and *N. fowleri* but not within *H. vermiformis*), but as well on the temperature. No strain could grow at 24°C and the maximum growth was observed in all cases between 30°C and 37°C. This study demonstrated the growth dependence to both temperature and host and supported the results obtained by studying the growth kinetics of 63 different *L. pneumophila* strains at different temperatures.

The impact of temperature on the virulence of bacterial pathogens is well stablished. In the case of bacterial pathogens of mammals, especially those that circulate often between environmental reservoirs and their warm-blooded hosts such as *Yersinia* species, elevated temperature (37°C) can indicate a successful infection of the host (43, 44). When bacteria sense the increase of temperature, virulence genes encoding type III secretion system, adhesins and other virulence factors are expressed (43, 45). The infectivity of the *L. pneumophila* strains has been shown previously (46, 47) to change according to the temperature. Edelstein et al. (1987) (46) demonstrated that this bacterium was about fivefold more virulent to guinea pigs when grown at 41°C than when it was grown at 25°C. Tachibana et al. (2013) (48) also showed that environmental strains isolated from a spa at 45°C were significantly more virulent than the clinical strain that they used as control.

4.5.2 Assessment of the virulence potential of environmental and clinical strains of *L. pneumophila* in a human macrophage-like cell line

L. pneumophila is an opportunistic pathogen that, besides parasitizing their natural host, is also capable to infect human cells by using a large set of effector proteins that have been acquired along the evolution with their natural amoeba hosts. A recent study has compared the genomes of 38 *Legionella* species and has identified in total nearly 6000 potential effector proteins (9). *L. pneumophila* is one of the species with a high number of species-specific effectors. Elucidating the role that each effector plays in the infection is usually complicated due to the high redundancy of effector functions. Next to highly sophisticated proteomic analysis, the in-vitro infection of host cells is a very usefull experimental approach to assess the virulence phenotype of *L. pneumophila* strains.

The understanding of *L. pneumophila* pathogenicity has been achieved mostly by the study of clinical isolates. Nevertheless, the analysis of the pathogenicity of environmental isolates is essential since environmental strains are considered the primary source of outbreaks as well as nosocomial and community acquired Legionnaires' disease (LD). In this study, an extensive dataset composed of 34 clinical and 51 environmental isolates was used to determine potential differences between environmental and clinical isolates in the interaction with one of their model host systems. Generally, the degree of virulence of the of *L. pneumophila* strains is determined by studying various traits such as cytotoxicity and intracellular multiplication within macrophages. Other approaches, as induction of apoptosis/DNA fragmentation, pore-formation-mediated cytolysis of the host, presence of the dot/icm loci and manifestation of specific serogroup or the virulence epitope MAb 3/1 are also used commonly (4, 49). Yet, none of these traits is conclusive. Moreover, specific strains are thought to be particularly pathogenic since they are frequently found among clinical isolates, although it is still not clear which specific

factors promote their pathogenicity. The assessment of the virulence of *L. pneumophila* strains is, nonetheless, very helpful in order to understand the epidemiology of these bacteria. The high resolution MLVA genotyping applied in this study to clinical and environmental *L. pneumophila* isolates allowed to improve the insights of the population structure of this pathogen in Europe (50) and offered an exhaustive view of the populations in the Middle East, especially of areas where no previous studies had been carried out. Therefore, to complement the genotyping results and better understand its epidemiological significance, the virulence of representative MLVA genotypes and the virulence at clonal level were assessed based in the cytotoxicity produced to human macrophages. In general, the virulence to human macrophages according different traits was assessed by using a diverse group of well geno- and physio-typed isolates to understand the importance of such traits.

Uptake, intracellular multiplication and cytotoxicity assays using THP-1 macrophages-like cells were used to examine the potential virulence of the *L. pneumophila* isolates. However, due to the high number of isolates to be tested and the greater effort and longer time required to test the growth kinetics for all isolates, a cytotoxicity assay based on absorbance measurements to indicate apoptosis was the approach selected for the final virulence assessment. Cytotoxicity was measured using four different multiplicity of infection (MOI) values (MOI 10, 25, 50 and 75) at 5 h and 24 h after infection. All 86 isolates tested showed significant levels of cytotoxicity in comparison to the *dotA* mutant of *L. pneumophila* Philadelphia-1, suggesting that all strains could cause infection to human cells in greater or lesser extent, probably due to the redundancy of effectors injected into the host cell. Cytotoxicity levels of all isolates increased proportionally with the increase of the MOI after 5 hours of incubation with Alamar blue, demonstrating the importance of the infection dose in the development of infection. At high MOI, i.e. MOI 75, the levels of cytotoxicity were generally very high for all strains tested and no significant differences could be detected, suggesting that inhalation of high doses of *L. pneumophila* could very likely cause infection independently of the inhaled strain.

Overall, the group of environmental isolates showed greater average cytotoxicity levels than the group composed of clinical isolates. In both groups, the range of relative cytotoxicity was very broad, ranging from 20% to 70%. Greater or equal pathogenicity to macrophages of environmental isolates in comparison to clinical isolates have been previously demonstrated (48, 51). Tachibana et al. (2013) (48) described the intracellular growth in THP-1 macrophages of several *L. pneumophila* isolates obtained from a spa in Japan. All of them presented significantly higher pathogenicity than the clinical reference strain.

The serogroup of the *L. pneumophila* isolates is one of the traits that was first determined after isolation in order to classified the isolates and determine their virulence potential. The serogroups of *L. pneumophila* are highly variable and their specificity is determined by its lipopolysaccharide (LPS) characteristics. *L. pneumophila* strains can be

classified into 15 different serogroups and monoclonal subgroups can be differentiated within serogroups 1, 4, 5 and 6 (52, 53). Genetic differences among the serogroups have been observed in the gene cluster regulating the biosynthesis of the O-antigen (54). The relation of the LPS and the pathogenicity of *L. pneumophila* strains is assumed since epidemiological data has shown that *L. pneumophila* serogroup 1 (sgp1), and particularly those strains carrying the virulence-associated LPS epitope recognized by monoclonal antibody (MAb) 3/1 positive, are the most common cause of LD in United States and Europe (55, 56).

In this study, the cytotoxicity to THP-1 human macrophages caused by sgp1 isolates did not differ to that caused by the rest of serogroups (non-sgp1). Moreover, the levels of cytotoxicity did not vary between the sgp1 isolates that presented the virulence epitope (MAb 1/3 positive) to those that did not present it (MAb 1/3 negtive). This high similarity between MAb 1/3 positive and MAb 1/3 negative strains was consistent with a previous study by Helbig et al. (2001) (57), where it was demonstrated that modifications in the LPS, although helpful as a typing tool, did not influence the virulence of the strains. The lack of differences in the levels of cytotoxicity between sgp1 and non-sgp1, which was contrary to what it could be expected, could be due to the high pathogenicity of the other serogroups used in the study (sgp3, sgp4, sgp8 and sgp2-14). Specifically, sgp3 has been reported to cause community-acquired pneumonia (58) and it is the second serogroup (3%) responsible for cases of legionellosis after sgp1 in Europe (19). Sgp4 and sgp8, although in much smaller proportions, have been also found to be responsible of community-acquired pneumonia cases and even a small outbreak (59). The fact that nine isolates in this study could not be sub-grouped to determine their specific serogroup and were enclosed in the group 2-14, makes a more accurate comparison between serogroups difficult. However, the four isolates from West Bank included in this group are likely to be sgp6, since all close genetically related isolates of their clonal complex (VACC11) were serotyped as sgp6. The rest five strains were isolated from the cooling tower at the HZI campus where only sgp4 and sgp6, besides sgp1, were obtained. Sgp6 is, after sgp1 and sgp3, the third serogroup (2%) found to cause legionellosis in Europe (19). Since sgp1 and non-sgp1 strain presented similar cytotoxicity to their host, the high clinical prevalence as well as the high rate of isolation of sgp1 from water could lead to hypothesize that sgp1 strains would have environmental advantages, as, for instance, increased virulence to their natural amoeba hosts. Messi et al. (2013) (20) demonstrated that out of a group of *L. pneumophila* strains of different serogroups isolated from tap water, the sgp1 strain multiplied more efficiently within *Acantamoeba polyphaga* than sgp6 and sgp9 strains. Nevertheless, the study carried out by Kahn et al. (2013) (54) revealed that sgp1 and sgp6 strains induced different immune responses, the sgp1 strains having a higher potential to disseminate through the blood stream and to lead to bacteremia.

Epidemiological studies have pointed out that, besides the high clinical prevalence of

sgp1 strains, few strains are responsible for most of the cases of legionellosis, suggesting differences in pathogenicity within sgp1 (5, 60). In this study, cytotoxicity levels to THP-1 macrophages varied significantly among different MLVA genotypes characterized as sgp1. The cytotoxicity of Gt75(49), genotype represented by clinical isolates, was significantly higher that the cytotoxicity exhibited by the clinical reference strains and the genotypes Gt4(17) and Gt6(18), both represented by environmental isolates and ST1. In order to rule out that clinical and environmental isolates of the same genotype presented distinct pathogenicity, the cytotoxicity of environmental and clinical isolates of Gt4(17) was compared and no significant differences were detected. Gt75(49) isolates were MAb 1/3 positive and corresponded to ST62. ST62 is the second most frequently found sequence type among clinical *L. pneumophila* serogroup 1 isolates in the Netherlands (61) and has been recurrently reported related to cases of legionellosis in other European and non-European countries (6, 60, 62). These results showed the high variability of the infectivity potential of genotypes with the same serogroup.

As presented in Chapter II and Chapter III the populations of *L. pneumophila* appeared to be naturally clonal and virulence traits could be linked to clonal complexes as it occurs in other microorganisms (63). Differences in the infectivity potential at clonal level were observed. VACC8 was the clonal complex that presented the highest average cytotoxicity in contrast to VACC2, which showed the lowest cytotoxicity level. VACC1, the largest and most diverse clonal complex analyzed in this study, showed a broad range of cytotoxicity suggesting high diversity in virulence potential of genotypes within the same clonal complex. The 85.7% of the isolates grouped in VACC1 and used in the analysis were sgp1 and the variability within this serogroup was also demonstrated. VACC8 was composed exclusively of 12 environmental isolates that belonged to serogroups 1(MAb negative), 4 and 8. They were isolated from biofilm in the West Bank and from the cooling tower at the HZI campus. VACC8 isolates belong to the so called "Lorraine lineage" because of the type strain of the cluster *L. pneumophila* Lorraine. The Lorraine strain, also known as ST47, is a highly virulent strain common in Europe. It is a significant cause of Legionnaires' disease in France, causing about 10% of the cases of culture-confirmed Legionnaires' disease (64). It has also been shown to have caused the 25% culture-confirmed community acquired cases of Legionnaires´ disease in England and Wales during 2000–2008 (5). Although most of the cases have been caused by Lorraine strains sgp1, this study revealed the high virulence potential of Lorraine strains with other serogroups. VACC2 is known as the "Philadelphia lineage". Although in this study VACC2 isolates (sgp1 and sgp6) showed reduced cytotoxicity, cooling tower isolates belonging to this clonal complex and characterized as sgp1 were responsible for two outbreaks occurring in 2000 and 2006 in Rennes, France. The last clonal complex whose pathogenicity was studied was VACC5, which consisted of sgp1 clinical isolates and sgp3 environmental isolates. Clinical sgp3 isolates from VACC5 have been reported in France (50), suggesting the capacity of causing disease of the members of

this VACC.

Nevertheless, despite the research efforts it is still not well understood what makes a strain more virulent than another. For instance, there are many uncertainties about the bacterial concentration required to result in a case of legionnaire's disease (LD) (65). In addition, in-vitro models of macrophage infections, as it was shown in the first part of this chapter, have confirmed that the infectivity potential of *L. pneumophila* strains can vary extensively. Especially, the infectivity potential varied between strains of serogroup 1. *L. pneumophila* serogroup 1 is the most commonly identified pathogen and is as well the most common serogroup isolated from the environment, as it was shown in Chapter II and Chapter III (5, 19). Its high occurrence in the environment may be related to the ability to replicate in a broader host range or to multiply more efficiently than other serogroups when the environmental conditions are suitable. Further research would be needed to better understand the ecological interactions between *L. pneumophila* and its in-situ hosts. Additionally, further research should take into consideration physiological studies to better understand the metabolism of different *L. pneumophila* strains. The relation between physiological traits and clonality would also deserve more attention, since a relation was found in this work between virulence potential and clonal complexes. Likewise, the relation between clonality and adaptation to different temperature ranges should be investigated.

As lLD is not transmitted from person to person, insights into the ecology of *L. pneumophila* may yield information that can be used to prevent the colonization of man-made freshwater systems by *L. pneumophila* strains. Understanding the ecology of this pathogen could help to determine methods for preventing its environmental dissemination and the transmission of legionellosis.

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4.7 Supplementary material

Table S1. List of *L. pneumophila* strains used in the infectivity and growth assays. Results of cytotoxicity at MOI 10 used in this study for comparisons between strains. Growth rates (μ) and maximum density (A) at each temperature tested.

						Growth in liquid medium													
						15ºC		22ºC		30ºC		37ºC		40ºC		43ºC			
Strain designation	**MLVA-8(12) genotype**	**Sgp-Mab[1]**	**MLVA CC[2]**	**Origin of isolate**	**% Cytotoxi-city (SD)[3]**	**μ[4] (h^{-1})**	**A[4] (LnOD600nm)**	**μ (h^{-1})**	**A (LnOD600nm)**	**μ (h^{-1})**	**A (LnOD600nm)**	**μ (h^{-1})**	**A (LnOD600nm)**	**μ (h^{-1})**	**A (LnOD600nm)**	**μ (h^{-1})**	**A (LnOD600nm)**	**Tº max. μ**	**Tº max. A**
A108	Gt10(93)	6 Dresden	VACC11	Env. WB	54.8 (6.6)	N.A.	N.A.	0.07	3.06	0.20	3.05	0.32	3.64	0.33	3.79	0.17	2.76	40	40
A112	Gt10(93)	6 Dresden	VACC11	Env. WB	51.1 (6.0)*	N.A.	N.A.	0.08	4.77	0.19	3.02	0.33	3.73	0.38	3.65	0.09	1.38	40	22
A114	Gt10(93)	6 Dresden	VACC11	Env. WB	67.2 (1.9) *	N.A.	N.A.	0.11	4.45	0.16	2.46	0.31	3.83	0.34	3.83	0.11	2.32	40	22
A127	Gt10(93)	6 Dresden	VACC11	Env. WB	62.6 (2.4) *	N.A.	N.A.	0.11	4.61	0.18	3.00	0.31	4.02	0.35	3.98	0.11	2.11	40	22
A14	Gt12(84)	8	Singleton	Env. WB	50.6 (9.8)	N.A.	N.A.	0.07	3.09	0.18	3.04	0.30	3.61	0.34	3.89	N.G.	N.G.	40	40
A15	Gt12(84)	8	Singleton	Env. WB	52.6 (4.5)	N.A.	N.A.	0.08	2.90	0.20	3.17	0.32	3.68	0.34	3.80	0.18	2.70	40	40
A17	Gt12(84)	8	Singleton	Env. WB	36.3 (2.5) *	0.04	3.67	0.09	3.94	0.22	4.18	0.31	3.69	0.35	3.60	NG	NG	40	30
A16	Gt12(84)	8	Singleton	Env. WB	41.8 (2.3) *	0.03	4.05	0.09	3.83	0.23	3.68	0.30	3.67	0.35	3.60	0.17	2.74	40	22
A18	Gt12(84)	8	Singleton	Env. WB	39.5 (1.0) *	N.A.	N.A.	0.12	4.55	0.19	4.31	0.35	4.18	0.35	4.04	0.04	0.37	40	22
H23	Gt14(30)	6 Chicago	Singleton	Env. G	N.A.	N.A.	N.A.	0.11	4.50	0.17	2.52	0.33	3.80	0.31	3.44	0.05	0.67	37	37
H1	Gt14(31)	6 Chicago	Singleton	Env. G	41.8 (2.2)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
H20	Gt14(31)	6 Chicago	Singleton	Env. G	41.2 (2.4)	N.A.	N.A.	0.07	2.95	0.17	3.00	0.28	3.77	0.37	3.75	0.22	2.37	40	37
H22	Gt14(31)	6 Chicago	Singleton	Env. G	41.8 (3.4)	N.A.	N.A.	0.07	3.04	0.18	2.95	0.28	3.73	0.37	3.70	0.33	2.70	40	37
H2	Gt14(31)	6 Chicago	Singleton	Env. G	40.4 (1.6)	0.03	3.76	0.10	4.05	0.21	3.87	0.30	3.66	0.33	3.59	0.04	0.64	40	22
H3	Gt14(31)	6 Chicago	Singleton	Env. G	43.9 (1.4)	0.04	3.81	0.09	4.15	0.19	3.98	0.31	3.81	0.31	3.71	N.G.	N.G.	40	22
H4	Gt14(31)	6 Chicago	Singleton	Env. G	55.8 (2.9) *	N.A.	N.A.	0.10	4.68	0.22	4.19	0.32	4.21	0.29	4.01	0.07	0.85	37	22
O100	Gt15(95)	1	VACC5	Env. Is	31 (0.8)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O58	Gt15(95)	3	VACC5	Env. Is	42.2 (1.2)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O59	Gt15(95)	3	VACC5	Env. Is	33.1 (1.4)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O60	Gt15(95)	3	VACC5	Env. Is	31.6 (1.2)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O61	Gt15(95)	3	VACC5	Env. Is	35.6 (2.3)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

H35	Gt22(102)	1	VACC6	Env. G	44.3 (5.6) *	0.03	3.72	0.09	4.24	0.21	4.26	0.30	3.90	0.36	3.97	0.11	1.70	40	30
O-H6	Gt22(99)	1	VACC6	Clin. Is	34.4 (1.3)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O-H8	Gt22(99)	1	VACC6	Clin. Is	31.7 (2.0)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
L11-082	Gt23(71)	1 Knoxville	VACC2	Clin. G	28.4 (1.2)	0.03	2.61	0.07	2.35	0.22	4.31	0.35	3.99	0.30	3.97	0.24	2.76	37	30
L11-219	Gt23(71)	1 Knoxville	VACC2	Clin. G	31.6 (1.8)	N.A.	N.A.	0.06	2.79	0.22	4.36	0.34	3.84	0.33	4.06	0.26	2.57	37	30
L03-315	Gt23(73)	1 Knoxville	VACC2	Clin. G	28 (9.5)	0.03	2.41	0.07	2.97	0.22	4.07	0.33	4.03	0.32	3.99	0.23	2.48	37	30
L09-183-1	Gt23(73)	1 Knoxville	VACC2	Clin. G	31.5 (4.5)	0.03	2.52	0.07	2.59	0.21	4.06	0.31	3.60	0.25	3.65	0.30	3.17	37	30
H42	Gt4(14)	1	Singleton	Env. G	50.3 (1.8) *	0.03	3.58	0.09	4.66	0.20	4.41	0.27	3.92	0.24	3.58	N.G.	N.G.	37	22
O123	Gt4(17)	1	VACC1	Env. Is	64.5 (7.2)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O128	Gt4(17)	1	VACC1	Env. Is	56.3 (4.4)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O55	Gt4(17)	1	VACC1	Env. Is	61.3 (4.5)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O72	Gt4(17)	1	VACC1	Env. Is	60.4 (3.4)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O75	Gt4(17)	1	VACC1	Env. Is	40.2 (2.7)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O-H10	Gt4(17)	1	VACC1	Clin. Is	30.3 (0.9)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O-H4	Gt4(17)	1	VACC1	Clin. Is	43.9 (1.9)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O-H9	Gt4(17)	1	VACC1	Clin. Is	27.5 (1.9)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
H50	Gt4(17)	4 Portland	VACC1	Env. G	58.3 (0.4)	N.A.	N.A.	0.05	2.27	0.15	2.96	0.28	3.61	0.23	3.69	0.12	1.25	37	40
H39	Gt4(17)	4 Portland	Singleton	Env. G	56.1 (4.4) *	N.A.	N.A.	0.09	3.49	0.19	3.16	0.25	3.74	0.24	3.59	N.G.	N.G.	37	37
H51	Gt4(17)	4 Portland	Singleton	Env. G	51 (4.5)	N.A.	N.A.	0.05	2.82	0.15	2.88	0.24	3.59	0.24	3.51	0.08	1.42	37	37
H16	Gt4(17)	(2-14)	Singleton	Env. G	34.3 (2.2) *	0.03	3.93	0.09	3.89	0.20	3.97	0.28	3.82	0.23	3.64	N.G.	N.G.	37	30
H38	Gt4(17)	1	Singleton	Env. G	37.1 (0.9) *	0.03	3.72	0.10	4.07	0.20	4.12	0.25	3.84	0.12	2.14	N.G.	N.G.	37	30
L03-610	Gt4(17)	1 OLDA	VACC1	Clin. G	43.1 (2.5)	0.03	2.42	0.08	3.01	0.29	3.97	0.31	3.70	0.33	3.58	0.21	2.78	40	30
L08-417	Gt4(17)	1 OLDA	VACC1	Clin. G	47.2 (0.9)	0.03	2.37	0.07	2.36	0.21	4.01	0.27	3.52	0.17	3.38	N.G.	N.G.	37	30
L08-498	Gt4(17)	1 OLDA	VACC1	Clin. G	24.2 (1.3)	0.03	2.46	0.07	2.52	0.22	4.08	0.30	3.54	0.21	3.59	0.25	3.57	37	30
L09-346	Gt4(17)	1 Allentown/ France	VACC1	Clin. G	53.9 (3.3)	0.03	2.54	0.06	2.50	0.24	4.61	0.33	3.66	0.34	3.84	0.13	1.06	40	30
L10-226	Gt4(17)	1 OLDA	VACC1	Clin. G	35.5 (1.2)	0.03	2.55	0.07	2.72	0.22	4.55	0.27	3.78	0.20	3.85	0.02	0.37	37	30
A1	Gt4(17)	1 OLDA	VACC1	Env. WB	30.8 (1.7)	0.03	4.18	0.10	4.26	0.20	4.04	0.26	3.67	0.12	1.80	N.G.	N.G.	37	22
A139	Gt4(17)	1	VACC1	Env. WB	17.3 (6.7)	N.A.	N.A.	0.10	3.97	0.20	3.37	0.21	3.87	0.23	2.42	N.G.	N.G.	40	22
A5	Gt4(17)	1 OLDA	VACC1	Env. WB	25.5 (3.9)	0.03	3.76	0.10	4.35	0.20	3.97	0.27	3.53	0.12	1.95	N.G.	N.G.	37	22
H40	Gt4(17)	4 Portland	Singleton	Env. G	49.2 (4.1) *	N.A.	N.A.	0.10	3.96	0.14	2.45	0.29	3.68	0.23	3.40	N.G.	N.G.	37	22
H72	Gt4(17)	1	Singleton	Env. G	31.8 (4.2) *	N.A.	N.A.	0.10	4.38	0.18	3.39	0.36	3.78	0.16	3.20	N.G.	N.G.	37	22
L. pn. Paris	Gt4(17)	1	VACC1	Clin.	27.9 (1.9)	0.03	3.67	0.11	4.36	0.20	4.14	0.32	3.82	0.19	3.53	N.G.	N.G.	37	22
A3	Gt4(20)	1 OLDA	VACC1	Env. WB	47.5 (3.3) *	0.03	4.43	0.08	4.49	0.20	3.91	0.25	3.66	0.12	1.70	N.G.	N.G.	37	22
H6	Gt5(11)	4 Portland	VACC1	Env. G	58.3 (4.0) *	N.A.	N.A.	0.10	4.78	0.26	4.52	0.37	4.37	0.35	4.37	N.G.	N.G.	37	22
H7	Gt5(12)	4 Portland	VACC1	Env. G	55.9 (1.7) *	N.A.	N.A.	0.09	4.56	0.21	4.25	0.32	4.30	0.20	4.03	N.G.	N.G.	37	22
L. pn Chicago-2	Gt52(105)	6	VACC2	Clin.	18.7 (0.9)	N.A.	N.A.	0.06	2.82	0.19	3.15	0.31	3.62	0.38	4.52	0.04	0.96	40	40

A121	Gt55(94)	6 Dresden	VACC11	Env. WB	N.A.	N.A.	N.A.	0.09	4.61	0.17	2.90	0.30	3.93	0.34	3.73	0.12	2.54	40	22
O28	Gt6(18)	1 OLDA	VACC1	Env. Is	51.7 (3.4)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O29	Gt6(18)	1	VACC1	Env. Is	25.7 (2.6)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O35	Gt6(18)	1	VACC1	Env. Is	38.8 (3.7)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O93	Gt6(18)	1	VACC1	Env. Is	43.4 (2.0)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O94	Gt6(18)	1	VACC1	Env. Is	40.1 (3.0)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O-H5	Gt6(18)	1	VACC1	Clin. Is	41.9 (0.5)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
O-H7	Gt6(18)	1	VACC1	Clin. Is	51.3 (2.8)	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.
L09-178	Gt64(114)	1 Knoxville	VACC2	Clin. G	23.5 (3.1)	0.03	2.46	0.07	2.77	0.21	4.25	0.31	3.59	0.29	3.67	N.G.	N.G.	37	30
L08-449	Gt64(115)	1 OLDA/Oxford	VACC2	Clin. G	20.2 (1.3)	0.02	2.35	0.06	2.65	0.24	3.78	0.32	3.35	0.36	3.50	N.G.	N.G.	40	30
L09-226	Gt64(115)	1 Knoxville	VACC2	Clin. G	30.6 (2.9)	0.03	2.59	0.06	2.72	0.23	4.48	0.31	3.62	0.29	3.67	0.07	1.29	37	30
L12-284	Gt64(115)	1 Knoxville	VACC2	Clin. G	21.1 (1.6)	0.03	2.63	0.12	4.57	0.23	4.26	0.29	3.67	0.34	4.03	0.24	2.94	40	30
A144	Gt64(72)	1	VACC2	Env. WB	55 (6.4) *	N.A.	N.A.	0.11	4.26	0.16	2.87	0.35	3.90	0.18	3.48	N.G.	N.G.	37	22
L. pn. Philadelphia-1	Gt64(74)	1	VACC2	Clin.	32.4 (3.7) *	0.03	3.60	0.11	4.30	0.22	4.22	0.25	3.84	0.12	2.08	0.11	1.86	37	22
H5	Gt7(26)	1 Bellingham	VACC1	Env. G	44.3 (3.0)	N.A.	N.A.	0.07	2.93	0.20	3.11	0.30	3.67	0.34	3.85	0.23	3.21	40	40
H13	Gt7(26)	1 Bellingham	Singleton	Env. G	47.4 (3.7)	N.A.	N.A.	0.06	2.89	0.19	3.26	0.31	3.68	0.27	3.64	0.19	2.95	37	37
H10	Gt7(26)	1 Bellingham	Singleton	Env. G	58.8 (7.3) *	0.04	3.53	0.08	4.20	0.22	4.04	0.32	3.49	0.34	3.63	N.G.	N.G.	40	22
H11	Gt7(26)	1 Bellingham	Singleton	Env. G	36.5 (1.8) *	N.A.	N.A.	0.12	4.53	0.25	4.15	0.37	4.11	0.36	4.19	0.06	0.77	37	22
H12	Gt7(26)	1 Bellingham	Singleton	Env. G	53.8 (2.6) *	N.A.	N.A.	0.11	4.56	0.23	4.20	0.34	4.23	0.37	4.23	0.04	0.43	40	22
L06-129	Gt71(135)	1 OLDA	VACC5	Clin. G	37.8 (1.8)	0.03	2.44	0.07	2.88	0.23	4.23	0.31	3.92	0.32	4.25	0.26	3.77	40	40
L03-095	Gt71(135)	1 Philadelphia	VACC5	Clin. G	42.1 (4.7)	0.02	2.59	0.07	2.79	0.24	4.18	0.33	3.80	0.35	3.77	0.29	3.38	40	30
L06-153	Gt71(135)	1 OLDA	VACC5	Clin. G	38.4 (3.6)	0.03	2.70	0.06	2.95	0.23	4.37	0.32	3.61	0.31	3.67	N.G.	N.G.	37	30
L09-329	Gt75(49)	1 Philadelphia	VACC1	Clin. G	52.6 (1.3)	0.03	2.61	0.06	2.25	0.23	4.50	0.32	3.58	0.31	3.78	0.08	0.71	37	30
L10-023	Gt75(49)	1 Knoxville	VACC1	Clin. G	72.7 (0.4)	0.02	2.18	0.05	1.98	0.25	4.53	0.32	3.59	0.33	3.86	0.28	3.09	40	30
L10-033	Gt75(49)	1 Knoxville	VACC1	Clin. G	67.9 (5.3)	0.02	2.33	0.06	2.26	0.24	4.38	0.30	3.69	0.33	3.92	0.15	3.27	40	30
L10-069	Gt75(49)	1 Knoxville	VACC1	Clin. G	54.8 (1.2)	0.02	2.23	0.07	2.36	0.25	4.08	0.33	3.62	0.30	3.97	0.24	2.89	37	30
L. pn. Corby	Gt86(97)	1	Singleton	Clin.	26.9 (8.2)	0.03	3.63	0.10	4.55	0.21	4.23	0.38	4.05	0.41	3.95	0.15	3.55	40	22
L. pn. Bloomington-2	Gt89(96)	3	VACC6	Clin.	34.1 (0.8)	N.A.	N.A.	0.12	4.28	0.23	3.20	0.33	3.94	0.37	3.90	0.16	2.59	40	22
L. pn. Los Angeles-1	N.T.	4	Singleton	Clin.	17.4 (2.8)	N.A.	N.A.	0.10	4.15	0.22	3.29	0.33	3.97	0.40	4.48	0.17	2.69	40	40
ΔdotA L. pneumohila Philadelphia-1 (negative control)					3.1 (2.1)*	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.	N.A.

[1] *Sgp-Mab:* serogroup and monoclonal antibody subgroup

[2] *MLVA CC:* MLVA Clonal complex

[3] Percentage of cytotoxicity at MOI 10 used in this study. *SD:* Standard deviation of replicates. MOIs 25, 50 and 75 were also analyzed for the same set of strains but data are not shown here. Asterisks indicate strains whose intracellular multiplication and uptake by THP-1 was studied.

[4] μ*:* Growth rate and [5]*A*: Maximum density.

N.T: not typeable; *N.A:* not assessed; *N.G*: no growth Env.: environmental, Clin.: clinical; WB: West Bank, G: Germany, Is: Israel

Figure S1. Principal coordinate analysis (PCO) of the growth rate (μ) of 37 environmental and 26 clinical *L. pneumophila* isolates growing at six different temperatures. Significant differences between environmental and clinical isolates were detected (ANOSIM R=0.206, P<0.001).

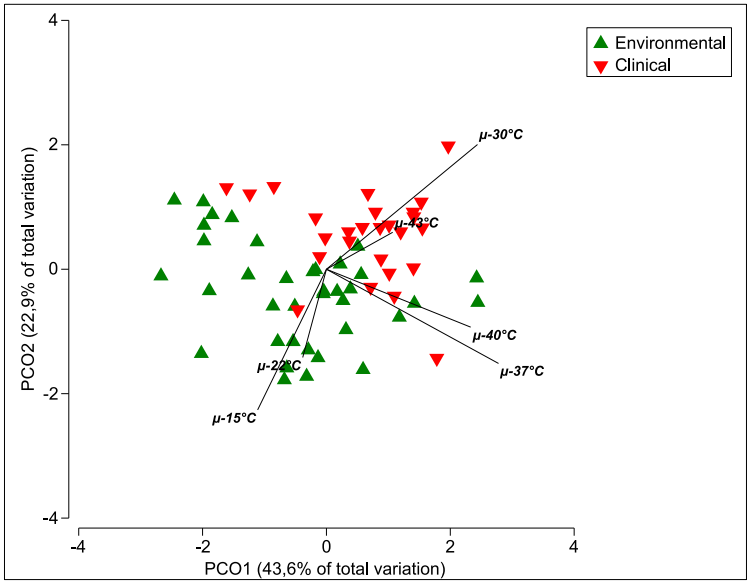


Figure S2. Principal coordinate analysis (PCO) of the maximal density (A) of 37 environmental and 26 clinical *L. pneumophila* isolates growing at six different temperatures. No differences were detected between environmental and clinical isolates by analyzing this growth parameter.

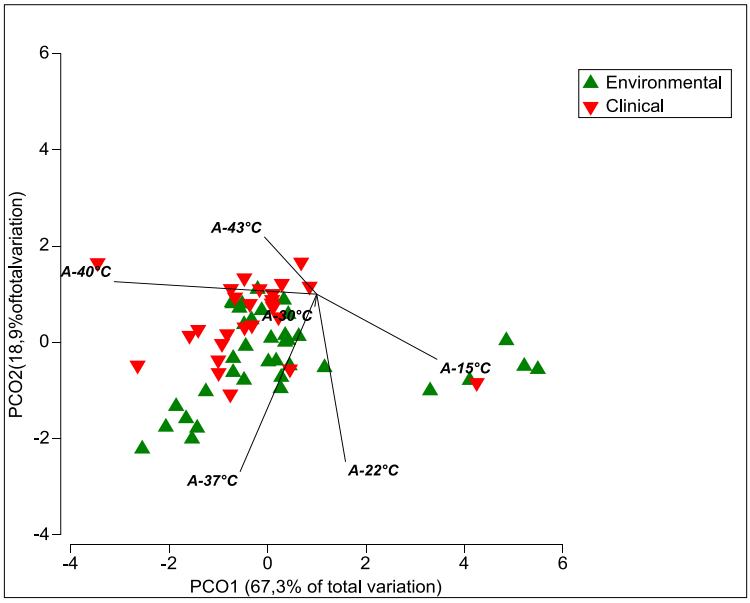


Figure S3. Principal coordinate analysis (PCO) of the growth rate (μ) of 37 environmental and 26 clinical *L. pneumophila* isolates growing at six different temperatures grouped according to their serogroup (sgp1 and non-sgp1). No significant differences were observed between the two groups.

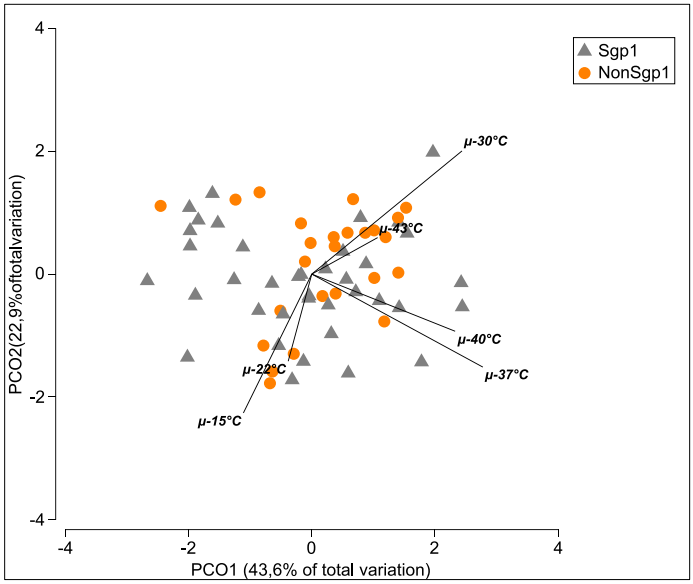


Figure S4. Principal coordinate analysis (PCO) of the growth rate (μ) and maximum density of 26 clinical and 37 environmental *L. pneumophila* strains isolated in Europe and the Middle East growing at six different temperatures. No differences were observed in the growth of environmental strains from the two distant geographic areas. (ANOSIM R=0.045, P>0.05).

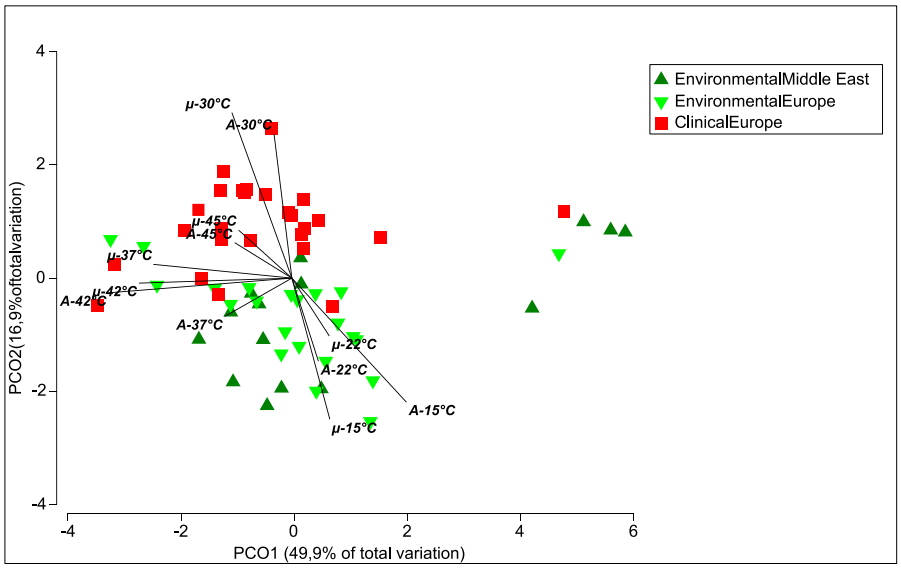
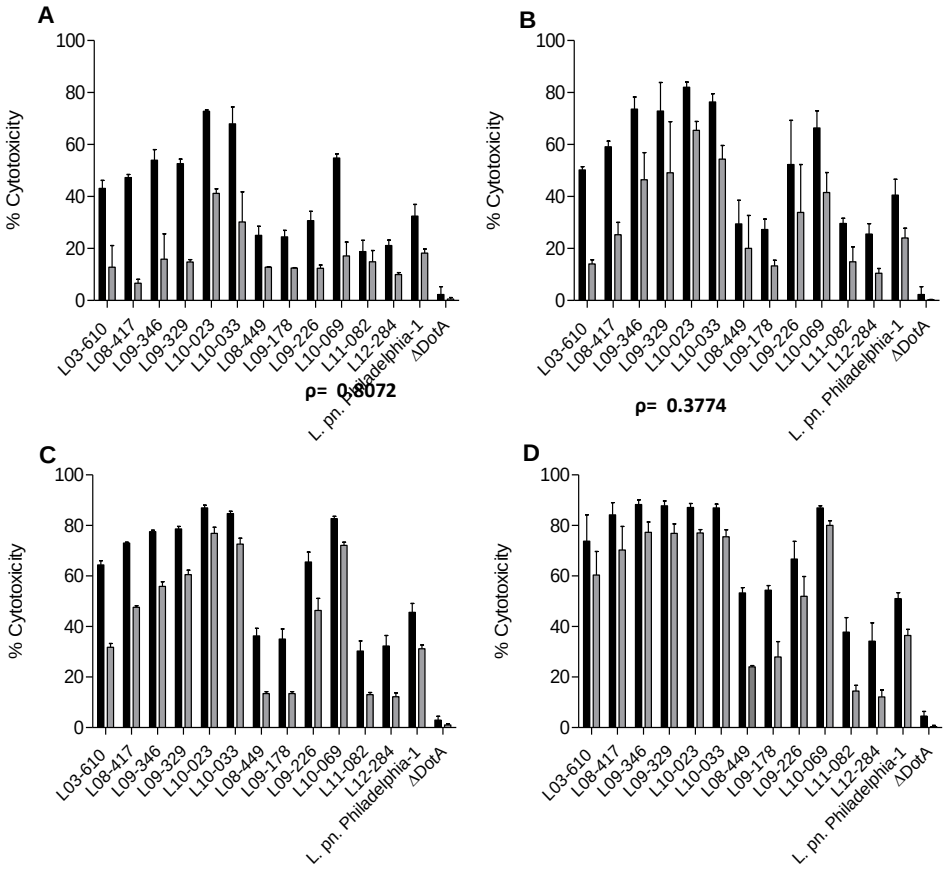


Figure S5. Cytotoxicity of a reduced group of 12 clinical *L. pneumophila* isolates after 5 h (black bars) and 24 h (grey bars) post infection of THP-1 human macrophages. Infections were carried out in triplicates. *L. pn.* Philadelphia-1 and *dotA* were used as positive and negative controls. **A**) MOI 10. **B**) MOI 25. **C**) MOI 50 and **D**) MOI 75.



5 Appendix

1. **Table A1.** List of *L. pneumophila* strains (n=611) isolated in Germany, Israel and West Bank analyzed in this study.
2. **Post-print of the article published in:**

   Rodríguez-Martínez S, Sharaby Y, Pecellín M, Brettar I, Höfle M, Halpern M. 2015. Spatial distribution of *Legionella pneumophila* MLVA-genotypes in a drinking water system. Water Res **77**:119–132. doi:10.1016/j.watres.2015.03.010

Table A1. List of *L. pneumophila* strains (n=610) isolated in Germany, Israel and West Bank analyzed in this study.

Strain	Sgp, mAb[1]	ST	ST profile[2]	MLVA-8(12)	MLVA-8(12) profile[3]	MLVA_CC	Country	Location	Sample type	Year
Berlin 10	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	1994
Berlin 13	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	1995
Berlin 15	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	1995
Berlin 16	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	1995
Berlin 2	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	1994
Berlin 3	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	1994
Berlin 6	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	1994
Berlin 7248/99	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	1999
Berlin 8	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	1994
Berlin 9	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	1994
Charite18398	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	2001
CharitéWV4205	1 Knoxville	182	3,4,1,3,35,9	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	2001
Freiburg 1	1 Bellingham	59	7,6,17,3,13,11	Gt22(64)	8,8,10,2,5,14,4,1,13,3,10,0,8	VACC6	Germany	Freiburg	Clinical	1999
H15	1			Gt4(14)	7,7,10,2,4,9,4,2,17,0,14,5,5	VACC1	Germany	Braunschweig	Environ.	2012
H18	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Germany	Braunschweig	Environmental	2013
H21	1 Oxford			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Braunschweig	Environmental	2013
H23	6 Chicago			Gt14(30)	7,8,8,2,4,14,2,1,18,3,0,4,8		Germany	Braunschweig	Environmental	2013
H26	10			Gt62(10)	7,0,10,2,4,9,4,2,17,0,14,5,5	VACC1	Germany	Braunschweig	Environmental	2013
H28	4 Portland			Gt4(14)	7,7,10,2,4,9,4,2,17,0,14,5,5	VACC1	Germany	Braunschweig	Environmental	2013
H29	4 Portland			Gt22(100)	8,8,10,2,5,14,4,1,13,2,10,0,8	VACC6	Germany	Braunschweig	Environmental	2013
H3	Chicago	1431	7,8,8,2,4,2,1	Gt14(31)	7,8,8,2,4,14,2,1,18,3,14,4,8		Germany	Braunschweig	Environmental	2009
H30	10			Gt49(101)	8,8,10,2,5,14,4,1,14,2,10,0,8	VACC6	Germany	Braunschweig	Environmental	2013
H31	(2-14)			Gt51(103)	8,8,11,2,5,14,4,1,13,3,10,0,8	VACC6	Germany	Braunschweig	Environmental	2013
H32	10			Gt22(102)	8,8,10,2,5,14,4,1,13,0,10,0,8	VACC6	Germany	Braunschweig	Environmental	2013
H35	1			Gt22(102)	8,8,10,2,5,14,4,1,13,0,10,0,8	VACC6	Germany	Braunschweig	Environmental	2013
H46	4 Portland			Gt43(104)	8,8,9,2,5,14,4,1,13,0,10,0,8	VACC6	Germany	Braunschweig	Environmental	2014
H47	10			Gt43(104)	8,8,9,2,5,14,4,1,13,0,10,0,8	VACC6	Germany	Braunschweig	Environmental	2014
H5	1 Bellingham	48	5,2,22,27,6,10,12	Gt7(26)	7,7,12,2,4,18,3,1,17,3,14,5,8	VACC1	Germany	Braunschweig	Environmental	2009
H50	4 Portland			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Braunschweig	Environmental	2014
H6	4 Portland			Gt5(11)	7,7,10,2,4,9,3,1,17,0,14,5,5	VACC1	Germany	Braunschweig	Environmental	2009
H61	(2-14)			Gt22(113)	8,8,10,2,5,14,4,1,13,0,10,5,8	VACC6	Germany	Braunschweig	Environmental	2014
H7	4 Portland			Gt5(12)	7,7,10,2,4,9,3,1,17,3,14,5,5	VACC1	Germany	Braunschweig	Environmental	2009
Hamburg P1-B	1 Philadelphia	332	7,10,17,6,14,11,3	Gt44(54)	8,8,10,2,0,14,4,1,13,3,6,0,8	VACC6	Germany	Hamburg	Clinical	1996
Hamburg P1-A	1 Philadelphia	332	7,10,17,6,14,11,3	Gt44(54)	8,8,10,2,0,14,4,1,13,3,6,0,8	VACC6	Germany	Hamburg	Clinical	1996
Heidelberg P5	1 Bellingham	334	2,6,17,6,13,11,11	Gt47(56)	8,8,10,2,4,14,4,1,6,3,10,0,8	VACC6	Germany	Heidelberg	Clinical	1996

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
L01-138	6 Chicago	437		Gt9(129)	9,8,11,2,4,13,3,4,17,3,6,4,8	VACC11	Germany	Schwedt	Clinical	2001
L01-284	1 Benidorm	387	2,6,17,14,13,11	Gt48(60)	8,8,10,2,4,15,3,1,5,3,9,0,8	VACC10	Germany	Görlitz	Clinical	2001
L01-354	1 Benidorm	34	3,13,1,25,14,9	Gt52(75)	8,8,11,2,5,16,1,1,3,3,6,4,8	VACC2	Germany	Hannover	Clinical	2001
L01-389	1 Knoxville	23	2,3,9,10,2,1,6	Gt36(45)	8,7,11,2,4,13,4,3,26,3,20,5,0	VACC3	Germany	Magdeburg	Clinical	2001
L01-403	1 Philadelphia	23	2,3,9,10,2,1,6	Gt36(45)	8,7,11,2,4,13,4,3,26,3,20,5,0	VACC3	Germany	Heidelberg	Clinical	2001
L01-409	1 Knoxville	182	3,4,1,3,35,9,11	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	2001
L01-443	1 Knoxville	9	3,10,1,3,14,9,11	Gt64(74)	8,8,11,2,4,16,1,1,3,3,6,4,8	VACC2	Germany	Herford	Clinical	2001
L02-034	10	440	3,10,1,28,14,9,1	Gt64(74)	8,8,11,2,4,16,1,1,3,3,6,4,8	VACC2	Germany	Görlitz	Clinical	2002
L02-456	1 Knoxville	182	3,4,1,3,35,9,11	Gt64(119)	8,8,11,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	2002
L02-465	1 Benidorm	425	2,10,3,15,9,4,11	Gt27(133)	10,8,8,2,5,13,2,2,18,3,10,4,8	VACC5	Germany	Berlin	Clinical	2002
L02-521	1 Philadelphia	62	8,10,3,15,18,1,6	Gt29(27)	7,7,12,2,4,15,2,2,22,3,20,5,0	VACC1-B	Germany	Bad Langensalza	Clinical	2002
L02-705	1 Benidorm	387	2,6,17,14,13,11,11	Gt48(60)	8,8,10,2,4,15,3,1,5,3,9,0,8	VACC10	Germany	Görlitz	Clinical	2002
L03-023	1 Knoxville	20	2,3,18,15,2,1,6	Gt33(40)	8,7,7,2,4,13,5,3,31,3,25,5,0	VACC3	Germany	Erlangen	Clinical	2003
L03-095	1 Philadelphia	62	8,10,3,15,18,1,6	Gt71(135)	10,8,8,2,4,13,2,2,17,3,10,4,8	VACC5	Germany	Saarbrücken	Clinical	2003
L03-315	1 Knoxville	182	3,4,1,3,35,9,11	Gt23(73)	8,8,10,2,4,17,1,1,3,3,6,5,8	VACC2	Germany	Frankfurt/Oder	Clinical	2003
L03-407	1 Benidorm	15	12,9,26,5,26,17,15	Gt66(131)	6,7,4,2,4,13,2,3,23,11,11,4,8		Germany	Frankfurt/Oder	Clinical	2003
L03-610 grün	1 OLDA	7	1,4,3,1,1,1,6	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Magdeburg	Clinical	2003
L03-610 rot	1 OLDA	7	1,4,3,1,1,1,6	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Magdeburg	Clinical	2003
L03-638	1 Bellingham	334	2,6,17,6,13,11,11	Gt47(56)	8,8,10,2,4,14,4,1,6,3,10,0,8	VACC6	Germany	München	Clinical	2003
L04-041	3	87	2,10,3,28,9,4,13	Gt30(137)	10,8,0,2,5,13,5,2,18,0,10,4,8		Germany	Kassel	Clinical	2004
L04-146	1 Knoxville	387	2,6,17,14,13,11,11	Gt48(60)	8,8,10,2,4,15,3,1,5,3,9,0,8	VACC10	Germany	Görlitz	Clinical	2004
L04-485	1 Knoxville	182	3,4,1,3,35,9,11	Gt82(124)	8,8,10,2,5,16,1,1,3,3,6,0,8	VACC2	Germany	Frankfurt/Oder	Clinical	2004
L04-497	1 Benidorm	407	6,10,15,3,21,14,9	Gt79(125)	8,8,8,2,5,12,4,1,24,3,6,0,8	VACC6	Germany	Frankfurt/Main	Clinical	2004
L04-545	6 Dresden	292	6,10,19,28,19,4,3	Gt40(47)	8,8,9,2,4,13,2,2,21,3,10,4,8		Germany	Heide-West	Clinical	2004
L04-598	6 Chicago	424	7,10,17,3,13,14,9	Gt82(127)	8,8,10,2,4,14,4,1,13,3,10,0,8	VACC6	Germany	Frankfurt/Oder	Clinical	2004
L05-228	1 Benidorm	46	5,1,22,5,6,10,12	Gt37(55)	8,7,12,2,4,19,4,1,16,17,12,5,8	VACC5	Germany	Cottbus	Clinical	2005
L05-341	6 Chicago	81	2,10,3,28,9,4,9	Gt8(132)	10,8,8,2,4,13,2,2,18,3,10,0,8	VACC1	Germany	Darmstadt	Clinical	2005
L05-362-1	1 Benidorm	6	1,4,3,1,1,1,15	Gt4(14)	7,7,10,2,4,9,4,2,17,0,14,5,5	VACC5	Germany	Kiel KH	Clinical	2006
L06-129	1 OLDA	169	6,10,3,10,9,4,6	Gt71(135)	10,8,8,2,4,13,2,2,17,3,10,4,8	VACC5	Germany	Brandenburg	Clinical	2006
L06-153	1 OLDA	169	6,10,3,10,9,4,6	Gt71(135)	10,8,8,2,4,13,2,2,17,3,10,4,8	VACC2	Germany	Brandenburg	Clinical	2006
L06-604	1 Knoxville	347	3,4,1,3,14,9,11	Gt54(123)	8,8,12,2,5,17,1,1,3,3,6,4,8	VACC6	Germany	Braunschweig	Clinical	2007
L07-002	1 Philadelphia	332	7,10,17,6,14,11,3	Gt82(126)	8,8,10,2,4,14,4,1,13,3,6,0,8		Germany	Gotha	Clinical	2007
L07-363	1 Knoxville	92	2,3,18,5,5,1,2	Gt80(65)	8,8,9,2,4,13,2,3,15,3,0,5,0	VACC1	Germany	Stuttgart	Clinical	2007
L07-551-1	1 Oxford/Phil.	1	1,4,3,1,1,1,1	Gt18(4)	7,7,7,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Hannover	Clinical	2007
L07-552-1	1 Philadelphia	1	1,4,3,1,1,1,1	Gt18(4)	7,7,7,2,4,9,4,2,17,3,14,5,5		Germany	Hannover	Clinical	2007
L07-590	1 Allentown/France	18	2,10,9,13,2,5,6	Gt73(51)	7,7,12,2,4,17,4,3,24,19,20,5,0	VACC8	Germany	Dresden	Clinical	2007

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
L07-667	1 Philadelphia	82	5,1,22,10,6,10,6	Gt37(46)	8,7,12,2,4,19,4,1,16,3,12,5,8	VACC2	Germany	Homburg	Clinical	2008
L08-147-1	1 Knoxville	182	3,4,1,3,35,9,11	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	2008
L08-147-2	1 Knoxville	182	3,4,1,3,35,9,11	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	2008
L08-148-1	1 Denver	182	3,4,1,3,35,9,11	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Clinical	2008
L08-148-2	1 Denver	182	3,4,1,3,35,9,11	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC3	Germany	Berlin	Clinical	2008
L08-217	1 Philadelphia	23	2,3,9,10,2,1,6	Gt36(45)	8,7,11,2,4,13,4,3,26,3,20,5,0	VACC1-A	Germany	Freiburg	Clinical	2008
L08-378	1 Philadelphia	1292		Gt69(67)	7,7,10,2,4,13,4,3,18,3,28,5,8	VACC1	Germany	Berlin	Clinical	2008
L08-417	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC2	Germany	Saarbrücken	Clinical	2008
L08-422-2	3	93	3,10,1,28,14,9,13	Gt86(118)	8,8,12,1,0,17,1,1,3,0,6,4,8		Germany	München	Clinical	2008
L08-444	1 OLDA	562		Gt34(136)	10,8,9,2,4,13,2,2,12,3,10,5,8	VACC2	Germany	Hannover	Clinical	2008
L08-449	1 OLDA/Oxford	561		Gt64(115)	8,8,11,2,4,17,1,1,3,3,6,4,8	VACC1	Germany	Stuttgart	Clinical	2008
L08-498	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC2	Germany	Greifswald	Clinical	2008
L08-532	1 Philadelphia	736		Gt53(85)	8,8,12,2,4,17,1,1,3,3,6,4,8	VACC2	Germany	Berlin	Clinical	2009
L09-178	1 Knoxville	1352		Gt64(114)	8,8,11,2,4,17,1,1,3,3,6,5,8	VACC2	Germany	München	Clinical	2009
L09-183-1	1 Knoxville	179	3,4,1,1,1,9,11	Gt23(73)	8,8,10,2,4,17,1,1,3,3,6,5,8	VACC6	Germany	Birkenfeld	Clinical	2009
L09-183-2	1 Benidorm	334	2,6,17,6,13,11,11	Gt81(128)	8,8,10,2,0,14,4,1,6,3,10,0,8	VACC2	Germany	Birkenfeld	Clinical	2009
L09-210	1 Knoxville	9	3,10,1,3,14,9,11	Gt52(122)	8,8,11,2,5,17,1,1,3,3,6,4,8	VACC2	Germany	Bad Nauheim	Clinical	2009
L09-226	1 Knoxville	737		Gt64(115)	8,8,11,2,4,17,1,1,3,3,6,4,8	VACC2	Germany	Hannover	Clinical	2009
L09-313	3	93	3,10,1,28,14,9,13	Gt84(116)	8,8,11,1,0,17,1,1,3,3,6,4,8	VACC1-B	Germany	Freiburg	Clinical	2009
L09-329	1 Philadelphia	62	8,10,3,15,18,1,6	Gt75(49)	7,7,13,2,4,15,2,2,22,3,20,5,0	VACC1	Germany	Trier	Clinical	2009
L09-346	1 Allentown/France	23	2,3,9,10,2,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC3	Germany	Freiburg	Clinical	2009
L09-415	1 OLDA	177	1,4,3,1,1,12,6	Gt36(45)	8,7,11,2,4,13,4,3,26,3,20,5,0	VACC3	Germany	Jena	Clinical	2009
L09-561	1 Oxford	23	2,3,9,10,2,1,6	Gt36(45)	8,7,11,2,4,13,4,3,26,3,20,5,0	VACC3	Germany	Freiburg	Clinical	2009
L09-624	1 Oxford	23	2,3,9,10,2,1,6	Gt36(45)	8,7,11,2,4,13,4,3,26,3,20,5,0	VACC2	Germany	Freiburg	Clinical	2010
L10-005	3	93	3,10,1,28,14,9,6	Gt24(117)	8,8,11,2,0,17,1,1,3,0,6,4,8	VACC1-B	Germany	Freiburg	Clinical	2010
L10-023	1 Knoxville	62	8,10,3,15,18,1,6	Gt75(49)	7,7,13,2,4,15,2,2,22,3,20,5,0	VACC1-B	Germany	Ulm	Clinical	2010
L10-033	1 Knoxville	62	8,10,3,15,18,1,6	Gt75(49)	7,7,13,2,4,15,2,2,22,3,20,5,0	VACC1-B	Germany	Ulm	Clinical	2010
L10-034	1 Knoxville	62	8,10,3,15,18,1,6	Gt67(22)	7,7,0,2,4,15,2,2,22,3,20,5,0	VACC1-B	Germany	Ulm	Clinical	2010
L10-069	1 Knoxville	62	8,10,3,15,18,1,6	Gt75(49)	7,7,13,2,4,15,2,2,22,3,20,5,0	VACC1-A	Germany	Ulm	Clinical	2010
L10-091	1 Philadelphia	435	2,10,18,15,2,1,1	Gt69(69)	7,7,10,2,4,13,4,3,18,3,30,5,8	VACC1	Germany	Erlangen	Clinical	2010
L10-163	1 OLDA	1	1,4,3,1,1,1,1	Gt68(19)	7,7,10,2,4,9,2,2,17,3,14,5,5	VACC1	Germany	Giessen-Marburg	Clinical	2010
L10-226	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC6	Germany	München	Clinical	2010
L10-273	1 Bellingham	59	7,6,17,3,13,11	Gt22(64)	8,8,10,2,5,14,4,1,13,3,10,0,8		Germany	Freiburg	Clinical	2010
L10-441	1 Allentown/France	224	4,8,11,16,42,12,2	Gt83(121)	8,8,11,2,0,9,2,1,8,3,0,4,0	VACC1	Germany	Ulm	Clinical	2010
L10-495	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1-B	Germany	Biedenkopf	Clinical	2010
L10-496	1 Philadelphia	62	8,10,3,15,18,1,6	Gt29(27)	7,7,12,2,4,15,2,2,22,3,20,5,0		Germany	Biedenkopf	Clinical	2010

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
L10-525	1 Allentown/France	44	4,8,11,10,10,12,2	Gt77(139)	9,8,15,2,4,8,2,2,17,15,0,4,0		Germany	Harburg	Clinical	2011
L11-002	1 Philadelphia	23	2,3,9,10,2,1,6	Gt36(45)	8,7,11,2,4,13,4,3,26,3,20,5,0		Germany	Ulm	Clinical	2011
L11-078	1 Philadelphia	47	5,10,22,15,6,2,6	Gt37(46)	8,7,12,2,4,19,4,1,16,3,12,5,8		Germany	Heidelberg	Clinical	2011
L11-082	1 Knoxville	182	3,4,1,3,35,9,11	Gt23(71)	8,8,10,2,4,17,1,1,3,2,6,5,8	VACC3	Germany	Greifswald	Clinical	2011
L11-124	1 Benidorm	42	4,7,11,3,11,12,9	Gt76(140)	7,8,9,2,0,14,3,1,12,3,0,4,8	VACC8	Germany	Köln	Clinical	2011
L11-154	1 Allentown/France	62	8,10,3,15,18,1,6	Gt29(27)	7,7,12,2,4,15,2,2,22,3,20,5,0	VACC2	Germany	Viersen	Clinical	2011
L11-198	1 Benidorm	8	1,4,3,1,1,1,9	Gt28(24)	7,7,11,2,4,9,4,2,17,3,14,0,5	VACC4	Germany	Hannover	Clinical	2011
L11-209	1 Knoxville	62	8,10,3,15,18,1,6	Gt29(27)	7,7,12,2,4,15,2,2,22,3,20,5,0	VACC1-B	Germany	Hannover	Clinical	
L11-219	1 Knoxville	182	3,4,1,3,35,9,11	Gt23(71)	8,8,10,2,4,17,1,1,3,2,6,5,8	VACC1	Germany	Oderland	Clinical	2011
L11-235	1 Philadelphia	46	5,1,22,5,6,10,12	Gt37(62)	8,7,12,2,4,19,4,1,16,16,12,5,8	VACC1-B	Germany	Münster	Clinical	2011
L11-285	1 Philadelphia	1	1,4,3,1,1,1,1	Gt28(25)	7,7,11,2,4,9,4,2,17,3,14,5,5	VACC2	Germany	Hannover	Clinical	2011
L11-413	1 Knoxville	9	3,10,1,3,14,9,11	Gt54(77)	8,8,12,2,5,16,1,1,3,3,6,4,8	VACC8	Germany	Regensburg	Clinical	2011
L11-462	1 Allentown/France	82	5,1,22,10,6,10,6	Gt37(62)	8,7,12,2,4,19,4,1,16,16,12,5,8	VACC1	Germany	Erlagen	Clinical	2011
L11-463	1 Allentown/France	224	4,8,11,16,42,12,2	Gt83(121)	8,8,11,2,0,9,2,1,8,3,0,4,0	VACC2	Germany	Erlagen	Clinical	2011
L11-483	1 Knoxville	9	3,10,1,3,14,9,11	Gt87(120)	8,8,12,2,1,16,1,1,3,3,6,4,8	VACC8	Germany	Leverkusen	Clinical	2011
L11-508	1 Philadelphia	1	1,4,3,1,1,1,1	Gt18(4)	7,7,7,2,4,9,4,2,17,3,14,5,5		Germany	Frankfurt/Main	Clinical	2012
L12-158	1 OLDA	1	1,4,3,1,1,1,1	Gt70(21)	7,7,10,2,2,9,4,2,17,3,14,5,5		Germany	Bochum	Clinical	2012
L12-230	6 Dresden	68	3,13,1,28,14,9,3	Gt24(116)	8,8,11,2,0,17,1,1,3,3,6,4,8	VACC2	Germany	Lübeck	Clinical	2012
L12-284	1 Knoxville	9	3,10,1,3,14,9,11	Gt64(115)	8,8,11,2,4,17,1,1,3,3,6,4,8	VACC1	Germany	Bochum	Clinical	2012
L12-296-	1 OLDA	45	5,1,22,26,6,10,12	Gt32(41)	8,7,7,2,4,19,4,1,12,3,12,5,8	VACC1	Germany	Göppingen	Clinical	2012
L12-317	1 Knoxville	444	2,10,22,10,2,1,6	Gt72(66)	7,7,10,2,5,13,4,3,18,3,28,5,8	VACC2	Germany	Lörrach	Clinical	2012
L12-360-	1 Allentown/France	82	5,1,22,10,6,10,6	Gt37(46)	8,7,12,2,4,19,4,1,16,3,12,5,8	VACC2	Germany	Zweibrücken	Clinical	2012
L12-384	1 Allentown/France	82	5,1,22,10,6,10,6	Gt37(46)	8,7,12,2,4,19,4,1,16,3,12,5,8	VACC8	Germany	Zweibrücken	Clinical	2012
L12-426	Dallas	1327		Gt78(130)	8,8,0,2,7,0,2,0,8,0,6,0,0	VACC1-A	Germany	Oberschleißeim	Clinical	2012
L12-435	1 Knoxville	9	3,10,1,3,14,9,11	Gt88(90)	8,8,12,2,8,17,1,1,9,3,6,4,8	VACC8	Germany	Mannheim	Clinical	2012
L12-480	1 Benidorm	42	4,7,11,3,11,12,9	Gt31(37)	7,8,9,2,5,12,3,1,12,3,0,4,0	VACC8	Germany	Kitzingen	Clinical	2012
L12-516	1 Bellingham			Gt85(138)	9,8,8,2,9,9,2,3,11,3,0,4,8		Germany	Heilbronn	Clinical	2012
L12-643	1 Allentown/France	788	2,6,17,14,2,8,11	Gt41(52)	8,8,9,2,4,15,3,1,5,11,9,0,8	VACC2	Germany	Regensburg	Clinical	2012
L12-654	1 Allentown/France	1403	2,1,22,16,6,10,26	Gt32(70)	8,7,7,2,4,0,4,1,12,3,12,5,8	VACC4	Germany	Freiburg	Clinical	2013
L13-236	1 OLDA	1	1,4,3,1,1,1,1	Gt4(59)	7,7,10,2,4,1,4,2,17,3,14,5,5		Germany	Stuttgart	Clinical	2013
L13-435	1 Knoxville	345	6,10,19,3,19,4,11	Gt39(48)	8,8,8,2,5,13,2,2,6,3,10,4,8	VACC10	Germany	Warstein	Clinical	2013
L13-440	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC8	Germany	Bochum	Clinical	2013
L13-443	3	93	3,10,1,28,14,9,13	Gt24(68)	8,8,11,2,0,16,1,1,3,0,6,4,8	VACC1	Germany	Warstein	Clinical	2013
M?chen 8	1 Bellingham	334	2,6,17,6,13,11,11	Gt47(56)	8,8,10,2,4,14,4,1,6,3,10,0,8	VACC5	Germany	München	Clinical	2003
W03-279	1 Bellingham	334	2,6,17,6,13,11,11	Gt50(57)	8,8,11,2,4,14,4,1,6,3,10,0,8	VACC1	Germany	Bottrop	Environmental	2006
W06-574	1 Benidorm	387	2,6,17,14,13,11,11	Gt48(60)	8,8,10,2,4,15,3,1,5,3,9,0,8	VACC2	Germany	Görlitz	Environmental	2006

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
W06-582	1 OLDA	387	2,6,17,14,13,11,11	Gt48(60)	8,8,10,2,4,15,3,1,5,3,9,0,8	VACC6	Germany	Coswig	Environmental	2006
W06-730-1	1 Knoxville	9	3,10,1,3,14,9,11	Gt53(76)	8,8,12,2,4,16,1,1,3,3,6,4,8	VACC6	Germany	Lörrach	Environmental	2007
W07-120 Stoma	1 Denver	182	3,4,1,3,35,9,11	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC10	Germany	Berlin	Environmental	2008
W08-297-	1 Knoxville	182	3,4,1,3,35,9,11	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Leuna	Environmental	2008
W08-444	1 Denver	182	3,4,1,3,35,9,11	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Berlin	Environmental	2008
W08-450	1 Bellingham	334	2,6,17,6,13,11,11	Gt47(56)	8,8,10,2,4,14,4,1,6,3,10,0,8	VACC6	Germany	München	Environmental	2008
W08-452	1 Bellingham	334	2,6,17,6,13,11,11	Gt47(56)	8,8,10,2,4,14,4,1,6,3,10,0,8	VACC6	Germany	München	Environmental	2009
W09-154	1 Benidorm	334	2,6,17,6,13,11,11	Gt47(56)	8,8,10,2,4,14,4,1,6,3,10,0,8	VACC6	Germany	Birkenfeld	Environmental	2009
W09-155	1 Benidorm	334	2,6,17,6,13,11,11	Gt47(56)	8,8,10,2,4,14,4,1,6,3,10,0,8	VACC6	Germany	Birkenfeld	Environmental	2009
W09-366	1 OLDA	182	3,4,1,3,35,9,11	Gt23(63)	8,8,10,2,4,16,1,1,3,3,6,5,8	VACC2	Germany	Frankfurt/Oder	Environmental	2010
W10-1075	1 Bellingham	334	2,6,17,6,13,11,11	Gt46(58)	8,8,10,2,4,14,4,0,6,3,10,4,8	VACC6	Germany	Ulm	Environmental	2010
W10-403	1 Knoxville	9	3,10,1,3,14,9,11	Gt52(75)	8,8,11,2,5,16,1,1,3,3,6,4,8	VACC2	Germany	Ulm	Environmental	2010
W10-921	1 OLDA	1	1,4,3,1,1,1,1	Gt4(61)	7,7,10,2,4,2,4,2,17,3,14,5,5	VACC1	Germany	Biedenkopf	Environmental	2011
W11-035	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Düsseldorf	Environmental	2011
W11-038	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Düsseldorf	Environmental	2011
W11-039	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Düsseldorf	Environmental	2011
W11-046	1 Bellingham	334	2,6,17,6,13,11,11	Gt47(56)	8,8,10,2,4,14,4,1,6,3,10,0,8	VACC6	Germany	Düsseldorf	Environmental	2011
W11-1006	1 OLDA	182	3,4,1,3,35,9,11	Gt54(56)	8,8,12,1,5,3,1,1,3,3,6,4,8	VACC2	Germany	Frankfurt/Oder	Environmental	2011
W11-1153-2	1 Philadelphia	1	1,4,3,1,1,1,1	Gt18(5)	7,7,7,2,4,4,4,2,17,2,14,5,5	VACC1	Germany	Frankfurt	Environmental	2011
W11-677-2	1 Benidorm	8	1,4,3,1,1,1,9	Gt28(24)	7,7,11,2,4,9,4,2,17,3,14,0,5	VACC1	Germany	Wolfenbüttel	Environmental	2011
W11-928	1 Philadelphia	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Hannover	Environmental	2012
W12-1082	1 Benidorm	42	4,7,11,3,11,12,9	Gt31(37)	7,8,9,2,5,12,3,1,12,3,0,4,0	VACC4	Germany	Kitzingen	Environmental	2012
W12-1190	1 Philadelphia	1	1,4,3,1,1,1,1	Gt18(4)	7,7,7,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Heilbronn	Environmental	2012
W12-1216	1 Allentown/France	788	2,6,17,14,2,8,11	Gt42(53)	8,8,9,2,4,15,3,1,10,11,0,0,0	VACC10	Germany	Regensburg	Environmental	2012
W12-1308	1 Allentown/France	1403	2,1,22,16,6,10,26	Gt32(70)	8,7,7,2,4,0,4,1,12,3,12,5,8	VACC8	Germany	Freiburg	Environmental	2012
W12-724	1 Philadelphia	1	1,4,3,1,1,1,1	Gt18(4)	7,7,7,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Kiel	Environmental	2012
W12-805	1 OLDA	45	5,1,22,26,6,10,12	Gt32(41)	8,7,7,2,4,19,4,1,12,3,12,5,8	VACC8	Germany	Göppingen	Environmental	2012
W12-948	1 Allentown/France	82	5,1,22,10,6,10,6	Gt37(46)	8,7,12,2,4,19,4,1,16,3,12,5,8	VACC8	Germany	Zweibrücken	Environmental	2013
W13-255	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Stuttgart	Environmental	2013
W13-845-1	1 Knoxville	600	6,10,19,28,19,14,11	Gt30(34)	7,8,8,2,5,12,2,2,11,3,0,4,8	VACC5-A	Germany	Warstein	Environmental	2013
W13-845-11	1 Knoxville	600	6,10,19,28,19,14,11	Gt30(33)	7,8,8,2,5,12,2,2,11,2,0,4,8	VACC5-A	Germany	Warstein	Environmental	2013
W13-845-13	10			Gt40(47)	8,8,9,2,4,13,2,2,21,3,10,4,8		Germany	Warstein	Environmental	2013
W13-845-16	Dresden			Gt56(86)	10,8,8,2,4,12,3,3,18,3,10,4,8		Germany	Warstein	Environmental	2013
W13-870-2	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Germany	Warstein	Environmental	2013
W13-872-2	1 Knoxville	600	6,10,19,28,19,14,11	Gt30(32)	7,8,8,2,5,12,2,2,11,3,15,4,8	VACC5-A	Germany	Warstein	Environmental	2013
W13-874-13	1 Knoxville	600	6,10,19,28,19,14,11	Gt30(35)	7,8,8,2,5,12,2,2,11,3,14,4,8	VACC5-A	Germany	Warstein	Environmental	2013

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
O10	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O100	3			Gt15(95)	9,8,8,2,5,13,2,2,21,0,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2013
O101	3			Gt15(96)	9,8,8,2,5,13,2,2,21,0,10,4,9	VACC5	Israel	Oranim Haifa	Environmental	2013
O102	3			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O103	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O104	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O105	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O106	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O107	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O108	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O109	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O11	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O110	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O111	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O112	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O113	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O114	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O115	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O116	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O117	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O118	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O119	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O12	1 OLDA			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O120	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O121	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O122	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O123	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O124	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O125	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O126	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O127	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O128	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O129	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O13	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O130	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O131	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
O132	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O133	1			Gt58(9)	0,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O134	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O136	1			Gt59(112)	0,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O138	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O139	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O14	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O140	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O141	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O142	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O144	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O145	1			Gt6(13)	7,7,10,2,4,9,4,2,18,2,17,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O146	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O147	1			Gt6(13)	7,7,10,2,4,9,4,2,18,2,17,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O148	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O149	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O15	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O150	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O151	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O153	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O154	1			Gt27(28)	10,8,8,2,5,13,2,2,18,0,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2013
O156	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O157	3			Gt57(29)	10,8,8,2,5,13,2,2,21,0,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2013
O159	3			Gt57(29)	10,8,8,2,5,13,2,2,21,0,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2013
O16	1			Gt6(83)	7,7,10,2,4,9,4,2,18,3,14,5,6	VACC1	Israel	Oranim Haifa	Environmental	2013
O160	3			Gt57(29)	10,8,8,2,5,13,2,2,21,0,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2013
O161	3			Gt57(29)	10,8,8,2,5,13,2,2,21,0,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2013
O165	3			Gt57(44)	10,8,8,2,5,13,2,2,21,3,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2013
O168	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O169	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O17	1 Oxford	1	1,4,3,1,1,1,1	Gt6(83)	7,7,10,2,4,9,4,2,18,3,14,5,6	VACC1	Israel	Oranim Haifa	Environmental	2012
O170	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O171	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O172	1			Gt6(13)	7,7,10,2,4,9,4,2,18,2,17,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O173	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O174	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
O175	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O176	1			Gt60(80)	7,0,7,2,0,9,4,2,30,3,14,5,0		Israel	Oranim Haifa	Environmental	2013
O177	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O178	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O18	1 Oxford			Gt6(83)	7,7,10,2,4,9,4,2,18,3,14,5,6	VACC1	Israel	Oranim Haifa	Environmental	2012
O180	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O181	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O182	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O183	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O187	NA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O188	NA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O189	NA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O19	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O190	NA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O191	NA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O192	NA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O193	NA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O194	NA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O195	NA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
O2	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O21	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O22	1 Oxford			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O23	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O24	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O25	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O26	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O28	1 OLDA	1	1,4,3,1,1,1,1	Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O29	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O3	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O30	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O32	1 Oxford	1	1,4,3,1,1,1,1	Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O33	1 Oxford			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O34	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O35	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O36	1 Oxford	1	1,4,3,1,1,1,1	Gt6(83)	7,7,10,2,4,9,4,2,18,3,14,5,6	VACC1	Israel	Oranim Haifa	Environmental	2012
O37	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
O38	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O39	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O4	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O40	1 Oxford			Gt6(83)	7,7,10,2,4,9,4,2,18,3,14,5,6	VACC1	Israel	Oranim Haifa	Environmental	2012
O41	1 Oxford			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O42	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O45	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O46	1 OLDA			Gt4(110)	7,7,10,2,4,9,4,2,17,3,14,5,6	VACC1	Israel	Oranim Haifa	Environmental	2012
O47	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O48	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O49	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O5	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O50	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O51	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O52	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O53	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O54	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O55	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O56	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O57	3			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O58	3			Gt15(95)	9,8,8,2,5,13,2,2,21,0,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2012
O59	3			Gt15(95)	9,8,8,2,5,13,2,2,21,0,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2012
O6	1			Gt4(110)	7,7,10,2,4,9,4,2,17,3,14,5,6	VACC1	Israel	Oranim Haifa	Environmental	2012
O60	3			Gt15(95)	9,8,8,2,5,13,2,2,21,0,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2012
O61	3			Gt15(95)	9,8,8,2,5,13,2,2,21,0,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2012
O63	3			Gt17(111)	7,7,8,2,3,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O64	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O65	1			Gt3(81)	7,7,10,2,4,9,4,2,0,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O66	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O67	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O68	1			Gt61(82)	7,0,10,2,4,9,4,2,0,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O69	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O70	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O71	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O72	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O73	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
O74	1			Gt4(110)	7,7,10,2,4,9,4,2,17,3,14,5,6	VACC1	Israel	Oranim Haifa	Environmental	2012
O75	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O76	1			Gt6(83)	7,7,10,2,4,9,4,2,18,3,14,5,6	VACC1	Israel	Oranim Haifa	Environmental	2012
O77	1			Gt3(81)	7,7,10,2,4,9,4,2,0,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O79	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O8	1			Gt4(110)	7,7,10,2,4,9,4,2,17,3,14,5,6	VACC1	Israel	Oranim Haifa	Environmental	2012
O80	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O81	1			Gt18(4)	7,7,7,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O82	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O83	1			Gt15(97)	9,8,8,2,5,13,2,2,21,3,10,4,8	VACC5	Israel	Oranim Haifa	Environmental	2012
O85	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O86	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O87	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O88	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O9	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O92	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O93	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O94	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O96	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O97	1			Gt3(81)	7,7,10,2,4,9,4,2,0,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2012
O99	3			Gt3(81)	7,7,10,2,4,9,4,2,0,3,14,5,5	VACC1	Israel	Oranim Haifa	Environmental	2013
OA1	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2013
OA10	1			Gt22(98)	8,8,10,2,5,14,4,1,13,3,10,4,8	VACC6	Israel	Alonei Aba House	Environmental	2012
OA11	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2012
OA12	1			Gt45(107)	8,8,10,2,0,16,1,1,3,2,6,4,8	VACC2	Israel	Alonei Aba House	Environmental	2013
O-A17	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2013
O-A19	1			Gt6(13)	7,7,10,2,4,9,4,2,18,2,17,5,5	VACC1	Israel	Alonei Aba House	Environmental	2013
O-A2	1			Gt22(99)	8,8,10,2,5,14,4,1,13,3,10,5,8	VACC6	Israel	Alonei Aba House	Environmental	2013
O-A23	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2013
O-A27	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2013
O-A28	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2013
OA3	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2012
OA4	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2012
OA5	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2012
OA6	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2012
OA7	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2012
OA9	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Alonei Aba House	Environmental	2012

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
O-H1	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Rambam Hospital	Clinical	NA
O-H10	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Rambam Hospital	Clinical	NA
O-H11	1			Gt20(115)	7,7,10,2,4,9,4,3,17,3,14,5,5	VACC1	Israel	Rambam Hospital	Clinical	NA
O-H2	1			Gt24(68)	8,8,11,2,0,16,1,1,3,0,6,4,8	VACC2	Israel	Rambam Hospital	Clinical	NA
O-H3	1			Gt19(17)	7,7,10,1,4,9,4,2,17,3,14,5,5	VACC1	Israel	Rambam Hospital	Clinical	NA
O-H4	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Rambam Hospital	Clinical	NA
O-H5	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Rambam Hospital	Clinical	NA
O-H6	1			Gt22(99)	8,8,10,2,5,14,4,1,13,3,10,5,8	VACC6	Israel	Rambam Hospital	Clinical	NA
O-H7	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Rambam Hospital	Clinical	NA
O-H8	1			Gt22(99)	8,8,10,2,5,14,4,1,13,3,10,5,8	VACC6	Israel	Rambam Hospital	Clinical	NA
O-H9	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Rambam Hospital	Clinical	NA
O-P11	1			Gt23(108)	8,8,10,2,4,16,1,1,3,3,6,4,8	VACC2	Israel	Pilot Technion	Environmental	2013
O-P1B2	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Yavniel	Environmental	2013
O-P1B3	(2-14)			Gt24(68)	8,8,11,2,0,16,1,1,3,0,6,4,8	VACC2	Israel	Yavniel	Environmental	2013
O-P1B4	1			Gt21(15)	7,7,10,1,4,9,4,2,18,2,14,5,5	VACC1	Israel	Yavniel	Environmental	2013
O-P3	1			Gt45(78)	8,8,10,2,0,18,1,1,3,2,6,4,8	VACC2	Israel	Pilot Technion	Environmental	2013
O-P5	1			Gt45(78)	8,8,10,2,0,18,1,1,3,2,6,4,8	VACC2	Israel	Pilot Technion	Environmental	2013
O-P6	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Pilot Technion	Environmental	2013
O-P7	3			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Pilot Technion	Environmental	2013
OT1	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
OT2	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
OT3	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T35	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T37	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T38	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Raz House	Environmental	2013
OT4	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T41	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T42	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T43	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T44	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T45	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T46	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T47	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T48	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
O-T49	1			Gt6(9)	7,7,10,2,4,9,4,2,18,2,17,4,5	VACC1	Israel	Raz House	Environmental	2013

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
OT5	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
OT6	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Raz House	Environmental	2013
OT7	2-14 (not 3)			Gt2(89)	7,8,3,2,4,9,1,0,11,2,12,4,5		Israel	Tamar House	Environmental	2013
OT8	2-14 (not 3)			Gt2(91)	7,8,3,2,4,9,1,0,11,3,12,5,0		Israel	Tamar House	Environmental	2013
OT9	2-14 (not 3)			Gt1(88)	7,8,3,2,4,9,1,2,11,2,12,5,0		Israel	Tamar House	Environmental	2013
O-W1	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Tiberias (Hila)	Environmental	2013
O-W10	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Mehanamia	Environmental	2013
O-W11	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Mehanamia	Environmental	2013
O-W12	1			Gt6(13)	7,7,10,2,4,9,4,2,18,2,17,5,5	VACC1	Israel	Mehanamia	Environmental	2013
O-W13	3			Gt25(7)	7,8,0,2,4,8,2,1,0,3,27,0,0		Israel	TIiberias (Shosh)	Environmental	2013
O-W14	1			Gt6(13)	7,7,10,2,4,9,4,2,18,2,17,5,5	VACC1	Israel	TIiberias (Shosh)	Environmental	2013
O-W15	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Tiberias (Hila)	Environmental	2013
O-W16	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Tiberias (Hila)	Environmental	2013
O-W17	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Tiberias (Hila)	Environmental	2013
O-W18	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Tiberias (Hila)	Environmental	2013
O-W19	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Tiberias (Hila)	Environmental	2013
O-W2	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Tiberias (Hila)	Environmental	2013
O-W20	3			Gt27(28)	10,8,8,2,5,13,2,2,18,0,10,4,8	VACC5	Israel	Arbel	Environmental	2013
O-W21	3			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Arbel	Environmental	2013
O-W22	3			Gt27(28)	10,8,8,2,5,13,2,2,18,0,10,4,8	VACC5	Israel	Arbel	Environmental	2013
O-W24	3			Gt27(2)	10,8,8,2,5,13,2,2,18,2,10,4,8	VACC5	Israel	Arbel	Environmental	2013
O-W25	3			Gt26(8)	7,8,8,2,4,8,2,1,3,3,14,5,5		Israel	TIiberias (Shosh)	Environmental	2013
O-W26	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	TIiberias (Shosh)	Environmental	2013
O-W27	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	TIiberias (Shosh)	Environmental	2013
O-W28	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Tiberias (Hila)	Environmental	2013
O-W29	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	Israel	Tiberias (Hila)	Environmental	2013
O-W30	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	Israel	Tiberias (Hila)	Environmental	2013
O-W31	3			Gt25(7)	7,8,0,2,4,8,2,1,0,3,27,0,0		Israel	Tiberias (Hila)	Environmental	2013
O-W33	1			Gt27(2)	10,8,8,2,5,13,2,2,18,2,10,4,8	VACC5	Israel	Arbel	Environmental	2013
O-W4	(2-14)			Gt23(108)	8,8,10,2,4,16,1,1,3,3,6,4,8	VACC2	Israel	Arbel	Environmental	2013
O-W5	3			Gt27(43)	10,8,8,2,5,13,2,2,18,0,11,4,8	VACC5	Israel	Arbel	Environmental	2013
O-W6	3			Gt27(28)	10,8,8,2,5,13,2,2,18,0,10,4,8	VACC5	Israel	Arbel	Environmental	2013
O-W7	3			Gt27(2)	10,8,8,2,5,13,2,2,18,2,10,4,8	VACC5	Israel	Arbel	Environmental	2013
O-W8	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Mehanamia	Environmental	2013
O-W9	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	Israel	Mehanamia	Environmental	2013
A1	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Ramallah	Environmental	2013

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
A10	6			Gt13(106)	8,8,11,1,4,16,1,1,3,3,6,4,8	VACC2	West Bank	Al Quds University	Environmental	2013
A100	1			Gt38(109)	8,8,6,2,4,8,1,1,3,2,6,4,8	VACC2	West Bank	Hospital F	Environmental	2013
A101	1			Gt63(83)	7,7,10,2,4,0,4,0,0,3,14,4,9	VACC1	West Bank	Hospital H	Environmental	2013
A102	1			Gt63(83)	7,7,10,2,4,0,4,0,0,3,14,4,9	VACC1	West Bank	Hospital B	Environmental	2013
A103	1			Gt63(83)	7,7,10,2,4,0,4,0,0,3,14,4,9	VACC1	West Bank	Hospital H	Environmental	2013
A104	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2013
A105	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2013
A106	1			Gt6(15)	7,7,10,2,4,9,4,2,18,2,14,5,5	VACC1	West Bank	Hospital G	Environmental	2013
A107	(2-14)			Gt9(92)	9,8,11,2,4,13,3,4,17,3,10,4,8	VACC11	West Bank	Hospital B	Environmental	2013
A108	6 Dresden			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2013
A109	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital F	Environmental	2013
A110	6 Dresden			Gt10(141)	9,8,6,2,4,13,3,4,10,3,10,0,8	VACC11	West Bank	Hospital F	Environmental	2013
A112	6 Dresden			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2013
A114	6 Dresden			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2013
A115	(2-14)			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2013
A116	6 Dresden			Gt10(141)	9,8,6,2,4,13,3,4,10,3,10,0,8	VACC11	West Bank	Hospital F	Environmental	2013
A119	6 Dresden			Gt64(74)	8,8,11,2,4,16,1,1,3,3,6,4,8	VACC2	West Bank	Hospital F	Environmental	2012
A12	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Al Quds University	Environmental	2012
A121	6 Dresden			Gt55(94)	9,8,6,2,4,13,3,0,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2012
A122	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2012
A123	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2013
A124	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A127	6 Dresden			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2012
A128	6 Dresden			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2012
A129	6 Dresden			Gt64(74)	8,8,11,2,4,16,1,1,3,3,6,4,8	VACC2	West Bank	Hospital F	Environmental	2012
A13	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Al Quds University	Environmental	2012
A130	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A131	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A132	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014
A133	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014
A134	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014
A135	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A137	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2013
A138	6 Dresden			Gt9(92)	9,8,11,2,4,13,3,4,17,3,10,4,8	VACC11	West Bank	Hospital B	Environmental	2012
A139	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A14	8	1358	5,2,22,10,6,25,203	Gt12(84)	7,7,9,2,4,17,1,1,18,0,14,5,8		West Bank	Al Quds University	Environmental	2012

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
A142	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital E	Environmental	2012
A143	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital E	Environmental	2012
A144	1			Gt13(72)	8,8,11,2,4,16,1,1,3,2,6,4,8	VACC2	West Bank	Hospital E	Environmental	2012
A148	6 Dresden			Gt10(141)	9,8,6,2,4,13,3,4,10,3,10,0,8	VACC11	West Bank	Hospital F	Environmental	2012
A149	6 Dresden			Gt10(141)	9,8,6,2,4,13,3,4,10,3,10,0,8	VACC11	West Bank	Hospital F	Environmental	2012
A15	8			Gt12(84)	7,7,9,2,4,17,1,1,18,0,14,5,8		West Bank	Al Quds University	Environmental	2012
A152	6 Dresden			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2012
A153	6 Dresden			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2012
A154	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital F	Environmental	2012
A156	6 Dresden			Gt64(74)	8,8,11,2,4,16,1,1,3,3,6,4,8	VACC2	West Bank	Hospital F	Environmental	2013
A157	6 Dresden			Gt10(141)	9,8,6,2,4,13,3,4,10,3,10,0,8	VACC11	West Bank	Hospital F	Environmental	2014
A159	6 Dresden			Gt10(141)	9,8,6,2,4,13,3,4,10,3,10,0,8	VACC11	West Bank	Hospital F	Environmental	2014
A16	8			Gt12(84)	7,7,9,2,4,17,1,1,18,0,14,5,8		West Bank	Al Quds University	Environmental	2012
A161	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital F	Environmental	2012
A162	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A163	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A164	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A165	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014
A166	(2-14)			Gt8(142)	10,8,8,2,4,13,2,2,18,3,10,4,8	VACC5	West Bank	Hospital G	Environmental	2012
A167	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A168	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A169	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2012
A17	8			Gt12(84)	7,7,9,2,4,17,1,1,18,0,14,5,8		West Bank	Al Quds University	Environmental	2012
A170	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2013
A171	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2013
A172	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2013
A173	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2013
A174	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2013
A175	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital C	Environmental	2013
A176	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital C	Environmental	2014
A18	8	1358	5,2,22,10,6,25,203	Gt12(84)	7,7,9,2,4,17,1,1,18,0,14,5,8		West Bank	Al Quds University	Environmental	2014
A180	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital F	Environmental	2014
A181	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital F	Environmental	2014
A182	6 Dresden			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2014
A183	6 Dresden			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2014
A184	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
A186	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014
A187	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014
A188	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014
A189	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014
A19	(2-14)	461	6,10,14,28,21,14,9	Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2012
A190	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014
A191	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014
A192	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2014
A193	6 Dresden			Gt40(47)	8,8,9,2,4,13,2,2,21,3,10,4,8	VACC5	West Bank	Hospital H	Environmental	2012
A194	6 Dresden			Gt40(47)	8,8,9,2,4,13,2,2,21,3,10,4,8	VACC5	West Bank	Hospital H	Environmental	2012
A195	6 Dresden			Gt40(47)	8,8,9,2,4,13,2,2,21,3,10,4,8	VACC5	West Bank	Hospital H	Environmental	2012
A196	(2-14)			Gt13(143)	8,8,11,1,4,16,1,1,3,0,6,4,8	VACC2	West Bank	Hospital E	Environmental	2012
A197	10			Gt13(143)	8,8,11,1,4,16,1,1,3,0,6,4,8	VACC2	West Bank	Hospital E	Environmental	2012
A198	(2-14)			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2012
A20	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital F	Environmental	2012
A21	(2-14)	461	6,10,14,28,21,14,9	Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2012
A22	(2-14)	461	6,10,14,28,21,14,9	Gt9(92)	9,8,11,2,4,13,3,4,17,3,10,4,8	VACC11	West Bank	Hospital H	Environmental	2012
A23	(2-14)	1438	7,10,3,28,9,14,3	Gt16(1)	9,8,8,2,4,13,2,2,18,3,10,4,8	VACC5	West Bank	Hospital G	Environmental	2012
A24	(2-14)			Gt8(23)	10,8,8,2,4,13,2,2,18,3,14,4,8	VACC5	West Bank	Hospital G	Environmental	2012
A25	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A26	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A27	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A28	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A29	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A3	1 OLDA	1	1,4,3,1,1,1,1	Gt4(20)	7,7,10,2,4,9,4,2,17,3,14,4,5	VACC1	West Bank	Al Quds University	Environmental	2012
A30	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A31	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A32	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A33	1			Gt6(18)	7,7,10,2,4,9,4,2,18,3,14,5,5	VACC1	West Bank	Hospital G	Environmental	2012
A140	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A141	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A145	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A177	(2-14)			Gt9(92)	9,8,11,2,4,13,3,4,17,3,10,4,8	VACC11	West Bank	Hospital B	Environmental	2014
A178	6 Dresden			Gt9(92)	9,8,11,2,4,13,3,4,17,3,10,4,8	VACC11	West Bank	Hospital B	Environmental	2014
A179	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2014
A2	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2012

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
A34	(2-14)			Gt16(1)	9,8,8,2,4,13,2,2,18,3,10,4,8	VACC5	West Bank	Hospital A	Environmental	2012
A35	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2012
A36	(2-14)			Gt16(6)	9,8,8,2,4,13,2,2,18,2,10,4,8	VACC5	West Bank	Hospital A	Environmental	2012
A37	(2-14)	1326	3,10,1,28,14,9,207	Gt13(72)	8,8,11,1,4,16,1,1,3,2,6,4,8	VACC2	West Bank	Hospital A	Environmental	2012
A38	(2-14)			Gt16(1)	9,8,8,2,4,13,2,2,18,3,10,4,8	VACC5	West Bank	Hospital A	Environmental	2012
A39	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2012
A4	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Al Quds University	Environmental	2012
A40	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2012
A41	(2-14)			Gt13(72)	8,8,11,1,4,16,1,1,3,2,6,4,8	VACC2	West Bank	Hospital A	Environmental	2012
A42	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2012
A43	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2012
A44	(2-14)			Gt13(72)	8,8,11,1,4,16,1,1,3,2,6,4,8	VACC2	West Bank	Hospital A	Environmental	2012
A45	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2012
A46	6 Dresden			Gt16(1)	9,8,8,2,4,13,2,2,18,3,10,4,8	VACC5	West Bank	Hospital A	Environmental	2012
A47	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2012
A48	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2012
A49	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2012
A5	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Al Quds University	Environmental	2012
A50	(2-14)			Gt16(1)	9,8,8,2,4,13,2,2,18,3,10,4,8	VACC5	West Bank	Hospital A	Environmental	2012
A51	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2012
A52	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2013
A53	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2013
A54	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2014
A55	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital A	Environmental	2014
A56	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2014
A57	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2014
A58	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2014
A59	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2014
A6	1 OLDA			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2012
A60	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2012
A61	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2012
A62	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2012
A63	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2012
A64	1	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2012
A65	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2012
A66	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2012

Strain	Sgp, mAb	ST	ST profile	MLVA-8(12)	MLVA-8(12) profile	MLVA_CC	Country	Location	Sample type	Year
A67	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital D	Environmental	2012
A68	(2-14)			Gt13(72)	8,8,11,1,4,16,1,1,3,2,6,4,8	VACC2	West Bank	Hospital D	Environmental	2012
A69	(2-14)			Gt13(72)	8,8,11,1,4,16,1,1,3,2,6,4,8	VACC2	West Bank	Hospital D	Environmental	2012
A7	8	1358	5,2,22,10,6,25,203	Gt11(87)	7,7,10,2,4,17,1,1,18,3,14,5,8		West Bank	Al Quds University	Environmental	2012
A70	(2-14)			Gt13(72)	8,8,11,1,4,16,1,1,3,2,6,4,8	VACC2	West Bank	Hospital D	Environmental	2012
A71	(2-14)	461	6,10,14,28,21,14,9	Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital C	Environmental	2012
A72	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital C	Environmental	2012
A73	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital C	Environmental	2012
A74	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A75	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A76	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A77	1			Gt4(16)	7,7,10,2,4,9,4,2,17,2,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A78	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A79	(2-14)	1326	3,10,1,28,14,9,207	Gt13(72)	8,8,11,1,4,16,1,1,3,2,6,4,8	VACC2	West Bank	Hospital B	Environmental	2012
A8	6	187	3,10,1,28,14,9,3	Gt84(106)	8,8,11,1,0,16,1,1,3,3,6,4,8	VACC2	West Bank	Al Quds University	Environmental	2012
A80	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A81	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2013
A82	(2-14)	461	6,10,14,28,21,14,9	Gt9(92)	9,8,11,2,4,13,3,4,17,3,10,4,8	VACC11	West Bank	Hospital B	Environmental	2013
A83	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2013
A84	(2-14)			Gt9(92)	9,8,11,2,4,13,3,4,17,3,10,4,8	VACC11	West Bank	Hospital B	Environmental	2013
A85	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2013
A86	(2-14)			Gt9(92)	9,8,11,2,4,13,3,4,17,3,10,4,8	VACC11	West Bank	Hospital B	Environmental	2012
A87	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A88	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A89	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A9	1 OLDA	1	1,4,3,1,1,1,1	Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Al Quds University	Environmental	2012
A90	1			Gt4(17)	7,7,10,2,4,9,4,2,17,3,14,5,5	VACC1	West Bank	Hospital B	Environmental	2012
A91	(2-14)	1482	7,10,3,28,9,4,207	Gt8(7)	10,8,8,2,4,13,2,2,18,2,10,4,8	VACC5	West Bank	Hospital E	Environmental	2014
A92	(2-14)	1482	7,10,3,28,9,4,207	Gt8(7)	10,8,8,2,4,13,2,2,18,2,10,4,8	VACC5	West Bank	Hospital E	Environmental	2012
A93	(2-14)			Gt24(68)	8,8,11,2,0,16,1,1,3,0,6,4,8	VACC2	West Bank	Hospital E	Environmental	2012
A94	(2-14)			Gt16(3)	9,8,8,2,4,13,2,2,18,2,10,5,8	VACC5	West Bank	Hospital E	Environmental	2012
A95	6 Dresden			Gt13(72)	8,8,11,2,4,16,1,1,3,2,6,4,8	VACC2	West Bank	Hospital E	Environmental	2012
A97	(2-14)			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2012
A98	6 Dresden			Gt13(72)	8,8,11,2,4,16,1,1,3,2,6,4,8	VACC2	West Bank	Hospital F	Environmental	2012
A99	6 Dresden			Gt10(93)	9,8,6,2,4,13,3,4,10,3,10,4,8	VACC11	West Bank	Hospital F	Environmental	2013
[1]Serogroup, monoclonal subtype										
[2] ST allele order: *flaA, pilE, asd, mip, mompS, proA, neuA*										

[3] MLVA allele order: *Lpms1, Lpms3. Lpms13, Lpms17, Lpms19, Lpms31, Lpms33, Lpms34, Lpms35, Lpms38, Lpms39, Lpms40, Lpms44*				